Interaction of *Staphylococcus epidermidis* with abiotic and biotic surfaces: a role in bone and wound infections

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ABSTRACT

Staphylococcus epidermidis has become one of the most important causes of nosocomial and foreign device-related infections. It causes osteomyelitis around orthopaedic implants and secrets surface associated proteins which have osteolytic activity. Adherence of *S. epidermidis* to biomaterials is a key factor in the development of a device-related infection. Colonisation of surgical sutures with bacteria has been suggested to increase the risk of wound infection and consequent complications. Recent studies have shown that *S. epidermidis* can persist inside macrophages.

The data presented in this thesis show that *S. epidermidis* is internalised by bone cells and epithelial cells and that the capacity of different strains to be internalised varied considerably. Use of a panel of inhibitors that block mammalian cell endocytic pathways showed that the process of internalisation of *S. epidermidis* by host cells was via a receptor mediated pathway. However there were differences in the pathways utilised by epithelial cells and bone cells to internalise *S. epidermidis*. The SdrG protein of *S. epidermidis* played a significant role in the internalisation of strain HB by bone cells and, to a lesser extent, by epithelial cells.

Investigation of the effect of *S. epidermidis* internalisation by bone cells on cytokine secretion showed that the uptake process did not induce higher levels of the secretion of four different cytokines (IL-6, IL-8, IL-1β and TNF-α). Additionally uptake of *S. epidermidis* did not induce apoptosis of bone cells, in contrast to what has been reported for *S. aureus*.

The capacity of *S. epidermidis* to adhere to surgical sutures constructed from different materials was strain dependent. Coating the sutures with human plasma increased the numbers of bacteria adhering to the sutures. A number of putative *S. epidermidis*

virulence factors, including the GehD lipase, SdrG, the fibronectin binding protein Embp and the autolysin AtlE were found to be involved in the adhesion of this bacterium to sutures.

The findings presented in this thesis contribute to our understanding of the interactions of *S. epidermidis* with abiotic surfaces and host cells.

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DEDICATION

To my parents and my wife, Ghadah, who have encouraged me throughout the time taken to complete this thesis.

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ABBREVIATIONS

agr	Accessory Gene Regulator
AtlE	Autolysin AtlE
ATP	Adenosine triphosphate
BMUs	Basic Multicellular Units
CFU	Colony Forming Unit
ClfA	Clumping Factor A
ClfB	Clumping Factor B
CNS	Coagulase Negative Staphylococci
CP/A	Capsular Polysaccharide Adhesin
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
Embp	Extracellular Matrix Binding Protein
GehC	GehC (Glycerol ester hydrolase C) lipase
GehD	GehD (Glycerol ester hydrolase D) lipase
GFs	Growth Factors
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
IL-12	Interleukin 12
IL-1β	Interleukin 1β
IL-6	Interleukin 6

IL-8	Interleukin 8
INF- γ	Interferon Gamma
JNK	c-Jun N-terminal Kinase
kDa	kiloDalton
KGF	Keratinocytes Growth Factor
MDC	Monodansylcadaverine
MMPs	Matrix Metalloproteinases
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
MSCRAMMs	Microbial Surface Components Recognising Matrix Molecules
NNIS	National Nosocomial Infections Surveillance System
NO	Nitric Oxide
OAF	Osteoblasts Activating Factor
OCIF	Osteoclast Inhibitory Factor
OD	Optical Density
OPG	Osteoprotegerin
OPGL	Osteoprotegerin Ligand
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDS	Polydioxanone
PIA	Polysaccharide Intercellular Adhesin
PTH	Parathyroid Hormone
RANK	Receptor Activator of NF-$_K$B
RANKL	Receptor Activator of NF-$_K$B ligand

rFnBPB	Recombinant fibronectin binding protein
rRNA	Ribosomal Ribonucleic Acid
SAPK	Stress-Activated Protein Kinase
sar	Staphylococcal Accessory Regulator
SEM	Surface Scanning Electron Microscope
TGF-α	Transforming Growth Factor- α
TNF	Tumor Necrosis Factor

Chapter 1

Chapter 1

General introduction

1.0 Staphylococci

1.1 Classification

Originally staphylococci were included together with other spherical bacteria under various classifications and names. Approximately 100 years ago, von Recklinghausen applied the term micrococci to gram-positive cocci observed in diseased tissue and pus from human abscesses (Evans and Brachman, 1998). Cohen in 1872 recognised that morphology alone was insufficient for classification. In 1883, Ogston introduced the name *Staphylococcus* for the group of micrococci causing inflammation and suppuration. He was the first to differentiate between *Staphylococcus* and *Streptococcus* (Balows et al., 1992). Rosenbach, in 1884 provided the formal description of *Staphylococcus*, dividing the genus into two species *Staphylococcus aureus* and *Staphylococcus albus*. In 1885, Passet added a third species *Staphylococcus citreus*. Flugge in 1886 rearranged the cocci and maintained the genus *Staphylococcus* separate from *Micrococcus*. He differentiated the two genera primarily on the basis of their action on gelatin and on relation to their host. Evans et al, in 1955 proposed separating micrococci and staphylococci on the basis of their relation to oxygen. The facultative cocci were placed in the genus *Staphylococcus* and obligate aerobes were placed in the genus *Micrococcus* (Balows et al., 1992).

In the 1960s, a clear distinction was made between staphylococci and micrococci on the basis of their DNA base composition. Molecular methods such as plasmid analysis, catalase sensitivity and recently genetic mapping are widely used (Alfred S Evans and Philips S Brachman, 1998; Poyart et al., 2001)

1.2 Ecology

Staphylococci are widespread in nature, they can be found on the skin, mucous membrane, pharynx, mouth, respiratory tracts and gastrointestinal tracts. The largest population of human staphylococci are found on the skin and mucous membranes surrounding openings to the body surface. Most knowledge concerning the ecology of staphylococci has been obtained by collectively sampling the surface, hair, and epidermal invagination of the skin using scrubbing or swabbing methods (Huebner and Goldmann, 1999). Staphylococci living in moist habitats may reach densities of 10^3 to 10^6 colony forming units per square centimetre (CFU/cm^2) while those occupying dry surfaces reach densities of 10 to 10^3 CFU/cm^2.

Some species and subspecies of staphylococci show a marked preference to certain habitats. For example, *S. auricularis* is one of the major species living in the adult external auditory meatus (Huebner and Goldmann, 1999). The coagulase positive *S. aureus* demonstrates preference for the anterior nares in adults (Kloos and Bannerman, 1994). The wide distribution of staphylococci over the body surface makes specimen collection a real challenge. Careful procedures should be used to isolate the organisms (Balows et al., 1992)

1.3 Coagulase negative staphylococci (CNS)

One of the methods used to differentiate staphylococcal species is the coagulase test, performed by adding the organism to rabbit's plasma. A test which shows clotting within 24 hours is considered positive. Until 1975, coagulase negative staphylococci were grouped together as *Staphylococcus albus* or *S. epidermidis*. The coagulase test grouped *S. aureus* as a coagulase positive species while *S. epidermidis* is coagulase negative. Coagulase negative staphylococci have long been considered as harmless skin commensals or contaminants with low pathogenic potential. Their role as pathogens was only really recognised in the 1970s (Williams et al., 1976).

Thirty two species of the genus *Staphylococcus* have been identified, fifteen of which are indigenous to human while the reminder are nonhuman pathogens. Data taken from the National Nosocomial Infection Surveillance (NNIS) system from January 1990 until May 1999 showed that coagulase negative staphylococci are the most commonly reported pathogen (37.3 % compared to 12.6 % for *S. aureus*) isolated from blood stream infections in intensive care unit patients (NNIS, 1999). It has also been reported that most staphylococci isolated from humans belong to the *S. saprophyticus* group or *S. epidermidis* group, which includes the species *S. epidermidis*, *S. capitis*, *S. hominis*, *S. haemolyticus*, *S. warneri*, *S. saccharolyticus*, *S. pasteuri* and *S. lugdunensis* (Huebner and Goldmann, 1999). *S. epidermidis* comprises 65-90 % of all staphylococci isolated from human sources while *S. hominis* is considered the second most common after *S. epidermidis* accounting for 6% of all isolated coagulase negative staphylococci from blood cultures (Weinstein et al., 1998). *S. saprophyticus* is considered an important pathogen in urinary tract infections of young women (Rupp et al., 1992).

1.3.1 Isolation and identification of CNS

The isolation of staphylococci from clinical specimens is a routine procedure in the clinical laboratory. It is usually not difficult, since staphylococci grow readily on commonly used media under a broad range of growth conditions. The initial identification is straight forward using conventional or automated methods. The three main methods for identification of CNS are: conventional laboratory techniques, commercial identification kits, and molecular biology-based methods. The determination of CNS to the species level through conventional testing is costly and time consuming. Rapid identification kits for CNS are commercially available. The accuracy of various rapid identification kits ranges from 70 to > 90 % (Kloos and Bannerman, 1994). Examples of these kits include Staph-Zym (Rosco, Taastrup, Denmark) and the Vitek system (GIP, bioMerieux Vitek, USA) (Lee and Park, 2001). Molecular biology-based methods include total genomic DNA-DNA hybridisation (Kloos and Bannerman, 1994), ribotype analysis , and polymerase chain reaction (PCR). Lee and Park have described the rapid species identification of coagulase negative staphylococci by rRNA spacer length polymorphism analysis (Lee and Park, 2001). The identification of CNS strains has become important since the recognition of their clinical significance. It is important in monitoring the distribution and involvement of these strains in nosocomial and other infections.

Identification of the etiological agent in a blood culture is a difficult task because of the possible contamination of samples with the commensal organisms. Bacteraemia accounts for 8% of all nosocomial infections according to the NNIS data. Coagulase negative staphylococci are responsible for one third of nosocomial bacteraemia (Costa et al.,

2004). Accuracy in diagnosing medical device bacteraemia is important because device removal may be necessary to eradicate the infection (Giamarellou, 2002) .

1.3.2 *Staphylococcus epidermidis*

S. epidermidis is the most commonly isolated staphylococcal species from human sources. Cells occur predominantly in pairs and tetrads but occasionally single cells are observed. It can be distinguished from *S. aureus* by its inability to produce coagulase. For a long time, it was considered as a non-pathogenic organism, but now is recognised as the most important pathogen in foreign body device infections. It rarely causes infection in the healthy host (except in the case of native valve endocarditis). Compromised hosts and patients with foreign devices or implants are more likely to be the target of *S. epidermidis* infections (Kloos and Bannerman, 1994). The diameter of *S. epidermidis* cells is 0.5-1.5 μm. Some strains can produce an extracellular material which enhances their adherence to and accumulation on the smooth surfaces of metal devices (Vuong and Otto, 2002).

S. epidermidis colonies are smooth, raised, glistening, circular and translucent or nearly opaque. Single colonies may reach 2.6- 6 mm in diameter on non selective media. With time and elevated temperature (above 35°C) or crowding, colonies develop depressed dark centres and become more sticky in consistency. The colonies of slime producing strains become very sticky. Small and slow growing colonies can be produced by cell wall-deficient strains. Most strains produce grey to greyish white colonies. Other rare strains may produce colonies that are yellowish, brownish or violet in colour (Balows et al., 1992).

1.3.2.1 *S. epidermidis* infections

S. epidermidis has been listed by the National Nosocomial Surveillance System, in 1998, as one of the most often isolated bacterial pathogens in hospital and one of the most important pathogens involved in a variety of infections such as nosocomial bloodstream infection and cardiovascular infections (NNIS, 1998). *S. epidermidis* infections are usually sub-acute or chronic because of the lack of severely damaging toxins in this organism (Vuong and Otto, 2002). Compared to *S. aureus*, *S. epidermidis* does not usually cause pyogenic infections in non-compromised patients, except in the case of native valve endocarditis (Kloos and Bannerman, 1994). Drug abusers and immunocompromised patients such as those under immunosuppressive therapy, AIDS patients, and premature newborns are highly susceptible to *S. epidermidis* infections.

There is a very strong relationship between the use of foreign body devices and *S. epidermidis* infections. This organism is the most important infective agent of foreign bodies, indwelling catheters and any implanted device (Schierholz and Beuth, 2001; Ferretti et al., 2003). Infection around these devices is usually difficult to treat especially if complicated by biofilm formation (Costerton et al., 1999; Stewart and Costerton, 2001). Infections of prosthetic cardiac valves are caused by coagulase negative staphylococci in 40-50 % of cases. Antibiotic treatment of these infections may require removal of the prosthetic valve (Huebner and Goldmann, 1999). *S. epidermidis* is considered the most common coagulase negative staphylococcal species causing native valve endocarditis (von Eiff et al., 2002), other species, such as *S. warneri* and *S. lugdunensis* have been implicated (Shuttleworth and Colby, 1992).

Despite the sub-acute presentation of native valve endocarditis serious complications often arise, including systemic embolisation and stroke, congestive heart failure, and disruption of valve leaflets (Burgert et al., 1999). *S. epidermidis* is reported to be the most common cause of endophthalmitis following penetrating eye injury. The infection rate is 15 % if foreign material is still present (Duch-Samper et al., 1997). Urinary tract infections caused by coagulase negative staphylococci are of two types. The first is caused by *S. saprophyticus*, which affects young females, and the other is a nosocomial infection, which is caused by *S. epidermidis* and affects mainly women (Ronald, 2002). *S. epidermidis* is also the predominant species in cases of cerebrospinal fluid shunt infections (Rotim et al., 1997). Diagnosis of these types of infections is usually made by Gram staining and culture of cerebrospinal fluid obtained from the shunt reservoir, or by lumbar puncture and cerebrospinal fluid microscopy and analysis of protein and glucose concentrations. The treatment includes shunt removal and replacement and antibiotic coverage using intraventricular vancomycin with or without rifampicin (Wang et al., 2004).

Coagulase negative staphylococci such as *S. epidermidis* are responsible for the majority of cases of chronic osteomyelitis associated with orthopaedic implants and 90% of pin tract infections (Kloos and Bannerman, 1994). Bacterial persistence is the rule in chronic osteomyelitis associated with a foreign body such as surgical plates and screws or joint replacement. In such cases antimicrobial therapy alone is often unsuccessful, and the infection is cured only by prosthesis removal and debridement of necrotic bone (Ciampolini and Harding, 2000). Three possibilities have been suggested by Nair et al, of how bacteria may cause pathological bone loss (Nair et al., 1996). Bacteria can directly

destroy the noncellular components of bone by liberating acid and proteases, promote cellular processes that stimulate bone degeneration or inhibit synthesis of bone matrix (Nair et al., 1996). An acute bacterial infection will cause an intense inflammatory response, thrombosis of endosteal and periosteal vessels, the bone then infarcts with subsequent abscess and sequestrum formation (Ciampolini and Harding, 2000). In the case of *S. epidermidis* infection, the inflammatory response is mainly sub-acute or chronic and could be related to the lack of severely tissue damaging toxins (Vuong and Otto, 2002). It remains to be determined how *S. epidermidis* induces bone destruction, but it could occur through a number of different routes including a reduction in bone matrix formation induced by the bacteria and/or the production of osteolytic material by the bacteria. Lerner et al, have shown that *S. epidermidis* can cause decreased bone matrix formation by osteoblasts *in vitro*, thereby suggesting that impaired osteogenesis might be an important element of osteomyelitis caused by this bacterium (Lerner et al., 1998). It has been shown that *S. epidermidis* surface proteins have potent osteolytic activity which can be blocked by neutralising antibodies to tumor necrosis factor α (TNF-α) and partially by high concentrations of interlukein-1β receptor antagonist (anti IL-1β) (Meghji et al., 1997).

1.3.2.2 *S. epidermidis* virulence factors

The word virulence derived from the Latin word "virulentus", meaning "full of poison" (Casadevall and Pirofski, 2001). It is currently used to characterise the relative capacity of a microbe to cause disease and has traditionally been used to describe microbial characteristics (Casadevall and Pirofski, 1999). In theory, any factor that enhances the fitness of a bacterium in its host can enhance virulence (Alksne and Projan,

2000). Casadevall and Pirofski recently proposed that virulence factors are microbial attributes that mediate host damage (Casadevall and Pirofski, 2001). *S. epidermidis* produces a very limited number of tissue damaging exoenzymes and toxins when compared to *S. aureus*. A cysteine protease and an extracellular metalloprotease of 32 kDa have been described in *S. epidermidis* (Teufel and Gotz, 1993). Two very similar lipases are found in *S. epidermidis*, which may be important in skin colonisation (Simons et al., 1998; Longshaw et al., 2000). A delta-like toxin consisting of 25 amino acid residues has been demonstrated in *S. epidermidis* (McKevitt et al., 1990). This was named alpha toxin and it is encoded by the *hld* gene located in the regulatory *agr* locus (Otto et al., 1998). This N-formylated alpha-helical peptide, alpha-toxin, causes the lysis of erythrocytes by forming pores in the cytoplasmic membrane (McKevitt et al., 1990). Purified alpha toxin has been shown to prevent *S. aureus* attachment to polymer surfaces (Vuong et al., 2000b).

Biofilm formation is considered as one of the important virulence factors of *S. epidermidis*. In the case of foreign device infection, two different stages have been described. The first is the primary attachment of bacteria to the material and the second stage involves the formation of multilayered cell clusters through cell-cell adhesion (O'Gara and Humphreys, 2001). *S. epidermidis* can attach directly to implant material or to host derived matrix proteins which have coated the device surface (Gottenbos et al., 2000). Direct attachment can be related to surface properties, such as hydrophobicity and surface charge. Indirect attachment could be by a bridging process in which bacteria attach to a host protein coating the surface. This may involve adhesins such as the **m**icrobial **s**urface **c**omponents **r**ecognising **a**dhesive **m**atrix **m**olecules (MSCRAMMs).

Lantibiotics are produced by *S. epidermidis* and other Gram-positive bacteria such as *Bacillus subtilis* (Fischetti et al., 2000). Lantibiotic production may play a substantial part in bacterial interference on skin and mucous membranes by excluding competing organism that are sensitive to their bactericidal activities (von Eiff et al., 2002). *S. epidermidis* expresses several iron-repressible cell-wall-associated and cytoplasmic associated proteins when isolated during infection in people, as well as when grown *in vivo* in experimental animal infections (Modun et al., 1998). These proteins include a 42 kDa protein that is a cell wall glyceraldehyde-3-phosphate dehydrogenase and functions as a receptor for human transferin (Modun and Williams, 1999), as well as a 32 kDa cytoplasmic membrane associated lipoprotein (Cockayne et al., 1998). These proteins may play a role in *S. epidermidis* virulence.

1.3.2.3 Virulence factor regulation

In *S. aureus*, the production of most extracellular and surface attached virulence factors is controlled by the accessory gene regulator (*agr*) (Morfeldt et al., 1996). The accessory gene regulator is part of a quorum-sensing system and its expression is cell density dependent. The *agr* locus is activated by an autoinducing peptide pheromone (AIP). The locus consists of two divergent transcriptional units, RNAII and RNAIII, which are under the control of the P2 and P3 promoters, respectively. RNAII is a polycistronic mRNA that encodes the *agrB* and *agrD* genes required for the synthesis of the AIP and also the two component signal transduction proteins, AgrA and AgrC, which are responsible for sensing and responding to the AIP. RNAIII is the effector molecule in the *agr* regulon acting primarily at the level of gene transcription (Novick, 2003). Generally, when cell density is low *agr* activity will be low, this allows the expression of

surface proteins which are important for colonisation by these bacteria. With an increase in cell density, *agr* activity will be stimulated by the accumulation of pheromones, which are post-translationally modified peptides produced by the *agr* system. Activation of *agr* will inhibit the production of surface proteins, and stimulate the release of extracellular degradative exoenzymes and toxins (Vuong and Otto, 2002). *S. epidermidis agr* mutants have been shown to have decreased expression of two extracellular virulence factors, a lipase and protease (Vuong et al., 2000a). Biofilm formation in *S. aureus* and *S epidermidis* has been reported to be influenced by the *agr* system. *S. aureus agr* mutants attach to polystyrene much better than *agr* positive strains. *S. epidermidis agr* mutants show an increased production of the major autolysin (AtlE) which mediates the primary attachment of this bacterium to hydrophobic surfaces (Vuong et al., 2000b).

Other regulatory loci such as the *sar* locus and the secondary sigma factor *sigB* locus have been found to affect virulence factor production and may also influence each other (Deora et al., 1997). Factors that can affect expression of gene in these regulatory loci are glucose and salt concentrations, pH, and oxygen pressure (Chan and Foster, 1998). Biofilm formation has been shown to be influenced by the global regulatory loci in *S. epidermidis* and *S. aureus*. Indeed, many of the products involved in biofilm development, including surface-associated adhesins and the autolysin AtlE (in *S. epidermidis*), are regulated by the *agr* system, at least *in vitro* (Yarwood and Schlievert, 2003). The *sigB* locus also influences biofilm formation (Knobloch et al., 2001).

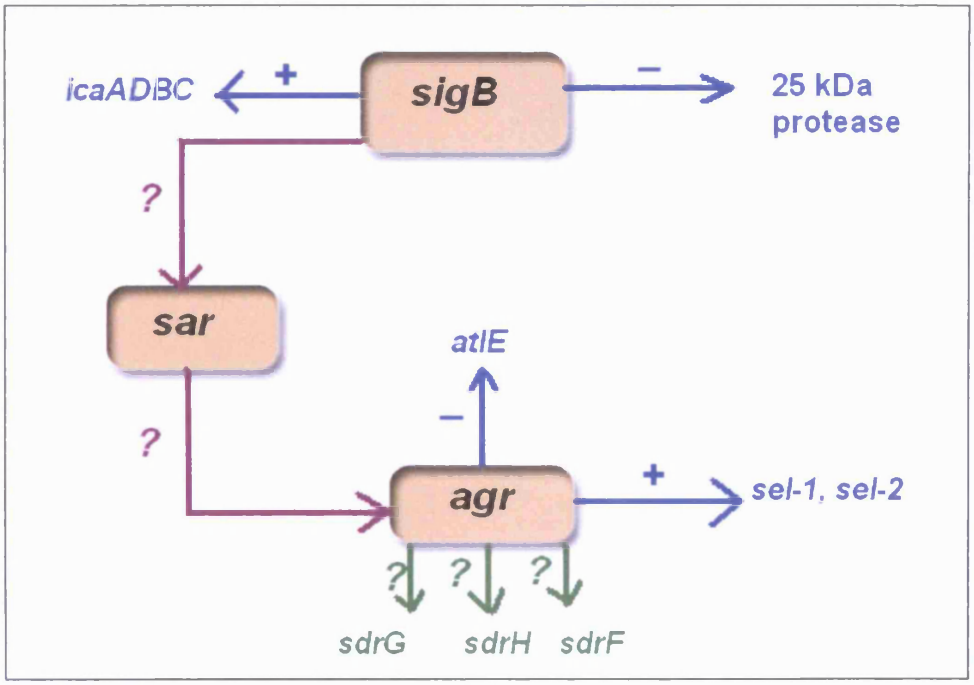

Figure 1-1: Model of global regulation of the expression of some of virulence genes by the regulators *agr*, *sar*, and *sigB*. Blue, published established interactions; green, suggested interactions; purple, interactions thought to occur by analogy to *S. aureus*. +, positive regulation; –, negative regulation. The diagram is a modification of that described by Vuong and Otto, 2002. *sel-1*: gene for the *S. epidermidis* GehC lipase; *sel-2*: gene for the *S. epidermidis* GehD lipase; *atlE*: gene for the *S. epidermidis* autolysin AtlE; *sdrG*: gene for the *S. epidermidis* fibrinogen binding protein; *sigB*: gene for the alternative sigma factor B; *sdrH* & *sdrF*: genes encoding the SD repeat region proteins SdrH and the SdrF; *sar*: staphylococcal accessory regulator gene; *agr*: accessory gene regulator; *icaADBC*: intercellular adhesion locus composed of four genes, *icaA*, *icaD*, *icaB* and *icaC*.

Although *agr*, *sar*, and *sigB* have all been found in *S. epidermidis*, it is unclear whether or not these loci fulfil the same tasks as in *S. aureus*. A global regulation model of some virulence gene expression in *S. epidermidis* can be seen in figure 1-1.

1.3.2.4 Microbial Surface Components Recognising Adhesive Matrix Molecules

The adhesion of bacteria to host cell surfaces involves bacterial surface components, called adhesins, that recognize and bind to host extracellular matrix (ECM) and cell surface molecules. Host ECM components such as fibronectin, collagen,

fibrinogen/fibrin, elastin, vitronectin and laminin have been reported to support bacterial adhesion (Joh et al., 1999). To be classified as an MSCARMM the molecule of interest must be localised to the microbial cell surface. Secondly, the microbial components must recognise a macromolecule ligand that can be found within extracellular matrix. Thirdly, the MSCRAMM's interaction with the extracellular matrix component should be of a high affinity and exhibit a high degree of specificity; i.e. unrelated molecules should not be able to significantly interfere with this interaction (Patti et al., 1994).

Specific *S. aureus* MSCRAMMs including fibronectin binding proteins, fibrinogen binding proteins, elasting binding adhesin and a collagen binding adhesin have been reported to be involved in adhesion of this bacterium to host cells (Alexander and Hudson, 2001). Apart from being important in bacterial adhesion some MSCRAMMs such as the fibronectin binding proteins have been shown to play an important role in the internalisation of some bacteria by host cells. It has been shown that *S. aureus* fibronectin binding proteins are required for the internalisation of this bacterium by endothelial cell (Peacock et al., 1999), epithelial cells (Dziewanowska et al., 1999) and osteoblasts (Ahmed et al., 2001).

Two MSCRAMMs have been identified and partially characterised in *S. epidermidis*, a fibrinogen binding protein (Fbe), known also as SdrG, which shows similarity to the *S. aureus* clumping factor A (Nilsson et al., 1998) and a fibronectin binding protein which has a ligand binding domain different from that found in the *S. aureus* FnBPs (Williams et al., 2002). The importance of both adhesins in virulence of *S. epidermidis* remains to be demonstrated.

1.3.2.5 *S. epidermidis* biofilms

Biofilms are highly ordered microbial communities enmeshed in a matrix suitable for survival of organisms either in multi- or mono-genus/species in a specific microniche (Watnick and Kolter, 2000). In the case of multi-species biofilms, the process of attachment of genetically distinct bacteria known as coaggregation has been suggested to influence the development of biofilms. The process of coaggregation occurs by two suggested routes. Single cells in suspension may specifically recognise and adhere to genetically distinct cells in the developing biofilm or coaggregation occurs in suspension of secondary colonisers followed by the subsequent adhesion to the developing biofilm (Rickard et al., 2003).

In recent years, the components of the matrix synthesised by staphylococci has in part been identified, and biochemical and genetic investigations have produced a much more defined idea of what a staphylococcal biofilm consists of and how it is produced. Most bacteria in natural environments are organised in biofilms (Watnick and Kolter, 2000). The development of a biofilm is initiated when bacterial cells attach to a surface and begin to secrete slime like substance, which serves to anchor the cells. Biofilm formation is thought to protect the organism from host defence mechanisms and antimicrobial agents (Hoyle and Costerton, 1991; Costerton et al., 1999; Stewart and Costerton, 2001). Further more, the altered physiology of cells results in altered growth rates which impair the effectiveness of growth-dependent antibiotics. In contrast, antibiotic therapy or the action of the host immune response, or both, is generally effective against individual cells released from the biofilm (Schwank et al., 1998). The presence of high bacterial counts,

and slow growth rates in biofilm environments may affect antibiotic activity (Konig et al., 2001).

S. epidermidis biofilms are proposed to form in a two-step process and much research has been directed towards understanding the molecular basis of this process. In the case of indwelling device biofilms a two-step model was proposed by Mack et al. (1994). Initial adherence involves the utilisation of a variety of factors such as the autolysin AtlE and polysaccharides followed by an accumulation process to form a mature biofilm. The primary attachment is also influenced by the physio-chemical properties of the material, hydrophobicity of bacterial surface and surface charge properties (Vuong and Otto, 2002). Other staphylococcal proteins such as staphylococcal surface proteins (SSP-1/SSP-2) have also been shown to be involved in the primary attachment (Fischetti et al., 2000). An extracellular polysaccharide called polysaccharide intercellular adhesin (PIA) has been reported to be involved in intercellular adhesion of bacteria (Rupp et al., 1999). The production of PIA was suggested to be an important factor in the next step of biofilm formation where accumulation occurs (Mack et al., 1994). Four unlinked gene loci related to biofilm formation and PIA production have been identified by transposon mutagenesis (Mack et al., 2000; Knobloch et al., 2001). The *ica* gene cluster, which contains all the genes necessary for production of PIA has been identified. *S. epidermidis* mutant with insertions in the *ica* locus have been shown to be impaired in the accumulative phase of biofilm formation (Mack et al., 2000). The ica locus is composed of the genes *icaA*, *icaD*, *icaB*, and *icaC*. IcaA is an *N*-acetylglucosaminyltransferase, which only achieves low activity without the presence of IcaD. IcaA and IcaD only produce *N*-acetyl oligomers of up to 20 residues of length. IcaC is responsible for the

production of PIA while the function of IcaB remains unclear (Vuong and Otto, 2002). The *ica* locus is also present in *S. aureus* and is important in biofilm formation for this organism (Cramton et al., 1999).

rsbU, which is the first gene in the *S. epidermidis sigB* operon of *S. aureus* has been shown to be involved in biofilm formation by *S. epidermidis* (Knobloch et al., 2001). Inactivation of *rsbU* inhibits biofilm formation and causes a severe reduction in PIA production by *S. epidermidis* strain 1457 but has no effect on the ability of this strain to bind to biomaterials (Knobloch et al., 2001). Cucarella et al, have described a novel protein in *S. aureus* named biofilm associated protein (Bap) involved in biofilm formation by this organism (Cucarella et al., 2001). The presence of ethanol or NaCl has been shown to affect biofilm formation and PIA production by *S. epidermidis*. Induction of PIA synthesis by NaCl depends on a functional *rsbU* gene while ethanol stress leads to induction of PIA synthesis and biofilm formation independent of *rsbU* (Knobloch et al., 2001). The regulatory mechanisms controlling expression of biofilm formation and PIA synthesis in *S. epidermidis* are only beginning to be unravelled and it is of primary importance to further characterise these mechanisms.

1.3.2.6 *S. epidermidis* and antibiotic resistance

Treatment of *S. epidermidis* infections requires the use of different antibiotics, some are very effective against these infections, but resistance to some of these antibiotics renders these infections difficult to treat. The antibiotics of first choice are the penicillin derivatives, such as methicillin and oxacillin. They are potent, bactericidal, and have shown good results in treating endocarditis due to coagulase negative staphylococci

(Gutschik, 1999). Cephalosporins like cefazolin and cefuroxime have excellent activity against methicillin susceptible staphylococcal infections, but more than 80 % of nosocomial *S. epidermidis* strains become resistant to methicillin (Vuong and Otto, 2002). Understanding the pattern of resistance and tolerance of *S. epidermidis* to antimicrobial agents is essential for the appropriate diagnosis and management of these infections.

Methicillin-resistant *S. epidermidis* isolates from patients are cross-resistant to all β-lactam agents *in vitro* (Miragaia et al., 2002). The prevalence of resistance has increased over the past decade. Mempel et al, have demonstrated that *S. epidermidis* isolates lacking the *mecA* gene can be methicillin resistant (Mempel et al., 1994). Quinolones such as ciprofloxacin were initially active against methicillin resistant *S. epidermidis*. However resistance to quinolones has developed due to the wide spread use of this type of antibiotic in the community as well as in hospitals (Sreedharan et al., 1991). Another factor that helped in the development of such resistance was the excretion of ciprofloxacin in sweat (Hoiby et al., 1997).

Vancomycin, which is a glycopeptide antibiotic, has been used as the principal treatment for methicillin resistant *S. epidermidis* infections, but as with all antibiotics, some resistance has developed against this drug (Dunne, Jr. et al., 2001). Although most clinical isolates of *S. epidermidis* are susceptible to vancomycin when tested *in vitro*, this is not the case *in vivo*. *S. epidermidis* that are highly susceptible to vancomycin show some tolerance to the antibiotic in biofilm. This tolerance was attributed to poor antibiotic penetration through the biofilm matrix (Costerton et al., 1999). However some studies

have shown that high vancomycin concentrations can be achieved in a biofilm environment but that this still fails to eradicate the organisms (Dunne, Jr. et al., 1993).

The development of glycopeptide resistance has led researchers to look for alternative drugs. Some drugs such as rifampicin, novobiocin and minocyclin have limited efficacy on their own in treating serious infections, but when used in combination with other antibiotics they show improved activity against these infections (Raad et al., 1998). Quinupristin/dalfopristin is a new semisynthetic streptogramin antibiotic that has good bactericidal activity against methicillin resistant *S. epidermidis*. The combination of quinupristin and dalfopristin has a synergetic bactericidal action (El Azizi et al., 2005). Other new drugs include the oxazolidinone antibiotics which are a novel class of antimicrobial agents that have been shown to be effective against most medically important Gram positive bacteria (Kaatz and Seo, 1996). Linezolid is the first member of the oxazolidinones, it is highly active against a number of Gram-positive isolates, including methicillin and methicillin/teicoplanin-resistant coagulase negative strains. Although linezolid concentrations in staphylococcal biofilms have been found to be lower than those of vancomycin, they caused a 91% reduction in biofilm-associated bacterial counts (Wilcox et al., 2001). Adler et al, have recently reported a teicoplanin-resistant small colony variant *of S. epidermidis* during vancomycin therapy which can cause persistent recurrent infections (Adler et al., 2003).

1.3.2.7 Adhesion of *S. epidermidis* to host cells

S. epidermidis can adhere to different host cells such as endothelial cells (Merkel and Scofield, 2001) and bovine mammary epithelial cells (Almeida and Oliver, 2001).

Bacterial adhesion can be by a direct interaction with the host cell or indirectly by binding to an already adhered substance such as host proteins or other bacteria. The adhesion process involves different mechanisms such as hydrophobic interactions, electrostatic forces, hydrogen bounding, cation bridging and receptor-ligand interactions (Henderson et al., 1999). Hydrophobic interactions have been termed non-specific and are balanced by the repulsive forces of the electrical double layer that surrounds both bacteria and tissue or material surfaces (Busscher et al., 1986).

Some bacteria may be able to produce a whole series of adhesive structures, which allow the organism to adhere to different host cells during the course of the infectious process in which it participates. Several polymeric carbohydrates and protein molecules have been proposed to be responsible for cell to cell adhesion in *S. epidermidis*. A 140-kDa protein called accumulation-associated protein (AAP) has been shown to play an important role in accumulative growth on polymer surfaces, but its function still needs to be investigated further (Hussain et al., 1997). Apart from PIA, another polysaccharide which has been described to be responsible for intercellular adhesion is poly-*N*-succinyl-glucosamine (PNSG) (McKenney et al., 2000). The cytoplasmic membrane of host cells contains molecules (receptors) that can be recognised by bacterial adhesins. Adhesion of *S. epidermidis* to host cells could occur directly to the lipid bilayer, or through cell surface receptors, or indirectly to molecules already adhered to the cell.

1.3.2.8 Adhesion of *S. epidermidis* to biomaterials

The application of implantable devices in medicine and dentistry has increased dramatically over the past twenty years. Devices such as arterial grafts, hip joints, and

dental implants have been approved for use on the basis of their host tissue biocompatibility and functional characteristics. Bacterial adhesion to biomaterials has been reported to be important in these device infections. The majority of biomaterial-associated infections, 40% - 75%, are caused by coagulase negative staphylococci, especially *S. epidermidis* (Boelens et al., 2000). Intravascular catheters which are essential in the management of patients in hospital are often left *in situ* for a long time. The major complication of the use of these catheters is infection, which is usually difficult to treat and may require the removal of the catheter. However, local and systemic factors play an important role in the host response to colonised foreign bodies such as implants and catheters.

A wide strain to strain variation in the capacity of *S. aureus* and *S. epidermidis* to adhere to implanted devices has been found. Slime producing strains of *S. epidermidis* adhere better than non slime producing strains (Garcia-Saenz et al., 2000). Device related infection rates vary according to the type and the location; however, Van de et al. (2001) has reported rates of 1% to 2% for orthopaedic implants (van de et al., 2001). Surface treatment of the device with different materials has been studied in the past. Precoating of different device surfaces with various plasma proteins has been shown to have an inhibitory effect on early adhesion of several *S. epidermidis* strains (Pei et al., 1999). Screening of 40 *S. epidermidis* strains has shown that while some strains can adhere to immobilised fibrinogen, the adherence of other strains was blocked by fibrinogen. It has been suggested that the difference in fibrinogen binding may be related to slime production (Baldassarri et al., 1997). Slime could mask the extracellular reactive adhesins or other molecules on the surface of *S. epidermidis* (Baldassarri et al., 1997). The gene

encoding the fibrinogen binding protein of *S. epidermidis* has been cloned (Nilsson et al., 1998; Hartford et al., 2001; Pei and Flock, 2001). A fibrinogen binding protein mutant of *S. epidermidis* has a decreased capacity to bind to immobilised fibrinogen (Pei and Flock, 2001). It has been reported that antibodies against the fibrinogen binding protein may protect against foreign device related infections caused by coagulase negative staphylococci (Rennermalm et al., 2004). The fibrinogen binding protein, SdrG, of *S. epidermidis* is more similar to the clumping factor B (ClfB) of *S. aureus* than to clumping factor A (ClfA), both of which have been shown to mediate adherence of *S. aureus* to fibrinogen (Ni et al., 1998).

Fibronectin is known to have heparin-binding domains, and is adsorbed differently to heparinized and non-heparinized polyvinyl chloride catheters. Precoating catheter surfaces with heparin renders the surface hydrophilic and reduces bacterial adhesion (Nomura et al., 1997). Fibronectin adsorption to biomaterial surfaces affects the binding of other proteins as well as microbes to these biomaterials (Yu et al., 1997). Williams et al, identified a fibronectin binding protein from *S. epidermidis* (Embp) and a recombinant protein which encompassed the fibronectin-binding domain of Embp could block binding of *S. epidermidis* to immobilised fibronectin (Williams et al., 2002).

1.3.2.9 Internalisation of *S. epidermidis* by host cells

Many species of bacteria have been shown to have the ability to enter and survive inside non phagocytic host cells (Hudson et al., 1995; Palmer et al., 1997; Menzies and Kourteva, 1998; Jett and Gilmore, 2002; Yavlovich et al., 2004). Two words have been used to describe the process, internalisation and invasion. Although they are sometimes

used interchangeably, they involve completely different processes. The word invasion means a hostile entrance into a domain of another. Bacteria and/or bacterial products may cause host cells to adapt actively and utilise their own structures for successful entry i.e. the process is driven by the bacteria. On the other hand the word internalisation means to take in or incorporate, this does not require activity from the bacterium and non vital organisms can be internalised.

The correct usage of these words requires a full understanding of the mechanisms by which bacteria enter the host cell. The entry of dead bacteria into bone cells which was described by Hudson et al. (1995) is an example of internalisation. In some cases a combination of invasion and internalisation can be found, and both bacteria and host cells play a part in the process. The entry process of the pathogen can be supported by the secretion of different bacterial extracellular proteins which act against host defence mechanisms.

One common element involved in the process of internalisation is the manipulation of normal host cell cytoskeletal components such as actin and tubulin resulting in invagination of the host cell membrane to enclose the bacterium within a vacuole. This often occurs by bacterial interference with host intercellular signalling pathways, either by stimulation or inhibition of signal transduction, or both. Almeida and Oliver (2001) have studied the interaction of three coagulase negative staphylococci species including *S. epidermidis* with bovine mammary epithelial cells. They have shown that bovine epithelial cells have been able to internalise selected coagulase negative species by a process that required the host cell cytoskeleton and protein kinase phosphorylation

(Almeida and Oliver, 2001). It has been also shown by electron microscopy that *S. epidermidis* can be internalised by endothelial cells and exist free in the cytoplasm. This has been suggested as a virulence factor associated with this bacterium's pathogenesis (Merkel and Scofield, 2001).

1.4 Bone

The skeleton is a highly specialised and dynamic organ that undergoes continuous regeneration. It consists of highly specialised cells, mineralised and unmineralised connective tissue matrix, and spaces that include the bone marrow cavities, vascular canals, canaliculi and lacunae. Bone is a part of the connective tissue components of the skeleton. It is formed from a combination of dense compact bone and cancellous (trabecular) bone.

1.4.1 Bone cells

There are different types of bone cells; these include osteoblasts, osteoclasts and osteocytes.

1.4.1.1 Osteoblasts

Osteoblasts are the cells found on bone surfaces and responsible for bone formation. Fully differentiated osteoblasts produce and secrete proteins that constitute the bone matrix. The matrix is subsequently mineralised under the control of the same cells. These cells are derived from primitive mesenchymal cells (Mackie, 2003) and are normally separated from the mineralised bone by a thin layer of unmineralised matrix called osteoid. Osteoblasts that become embedded in the bone matrix differentiate into osteocytes. Apart from being bone forming cells, osteoblasts play a role in bone

resorption through the regulation of activity and differentiation of the bone-resorbing osteoclasts (Manolagas, 2000).

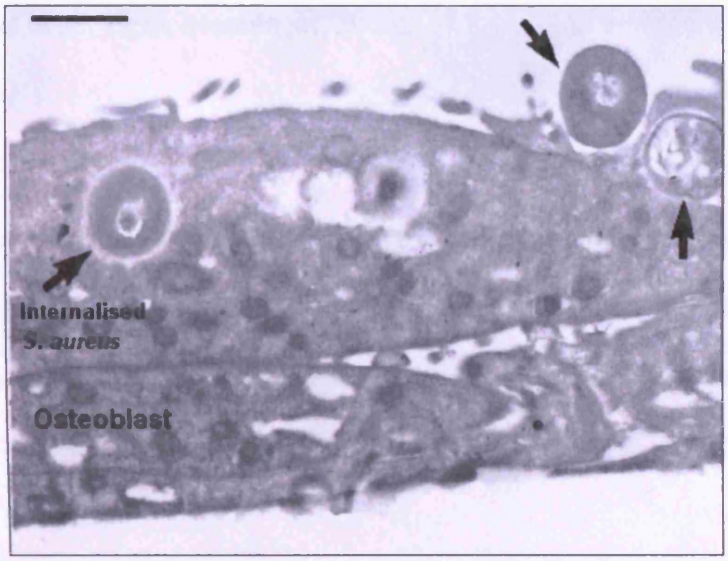

Figure 1-2: Transmission electron micrograph of *S. aureus* infected osteoblasts. Bar represents 1μm. *S. aureus* internalised by, or associated with osteoblast are arrowed. The image is a modification of that described by Jevon et al, 1999.

It has been reported that osteoblasts can internalise bacteria such as *S. aureus* (figure 1-2) (Hudson et al., 1995; Bost et al., 1999; Jevon et al., 1999; Reilly et al., 2000; Ahmed et al., 2001). Internalisation of bacteria by osteoblasts has been suggested to play a role in the chronicity of bone infection (Hudson et al., 1995).

1.4.1.2 Osteoclasts

Osteoclasts are multinucleated cells with abundant mitochondria, numerous lysosomes, and free ribosomes. They are derived from fusion of mononuclear haemopoietic precursors (Mackie, 2003). Osteoclasts are usually large (50- 100 μm in diameter) and secrete matrix metalloproteinases, and cathepsins into the area of bone

resorption. One of their features is the presence of high amounts of acid phosphatase, an iron-containing phosphohydrolase enzyme, tartrate-resistant acid phosphatase (TRAPase). This feature is commonly used for the detection of osteoclasts in bone specimens (Udagawa et al., 1990; Manolagas, 2000).

1.4.1.3 Osteocytes

Osteocytes are the most abundant cell type in bone. Osteocytes are regularly spaced throughout the mineralised matrix and communicate with each other and with cells on the bone surface via multiple extensions of their plasma membrane that run along the canaliculi.

1.4.2 Apoptosis of bone cells

Apoptosis is a programmed cell death that is considered as a fundamental cellular mechanism (Marouni and Sela, 2004). It is characterised by a series of changes including DNA fragmentation, nuclear condensation and membrane blebbing. Many bacterial pathogens are capable of inducing host cell death via apoptosis (Grassme et al., 2001). Apoptosis plays a critical role in bone development and growth and in the turnover of mature bone (Atkins et al., 2002). Bacteria can induce host cell apoptosis by a number of mechanisms such as the secretion of protein synthesis inhibitors, pore forming proteins or activation of endogenous death processes (Wesson et al., 2000). Apoptosis has both advantages and disadvantages. An example of the advantages of apoptosis is when a normal host cell detects abnormal mitogenic stimulation, and it responds by preventing further division through a programmed cell death. Examples of the disadvantages include apoptosis of macrophages which may protect the bacterium against phagocytosis

(Grassme et al., 2001) and decreased osteoclast apoptosis which may lead to increased bone loss (Xing and Boyce, 2005).

The average life span of human osteoclasts is about 14 days, while the average lifespan of osteoblasts is 90 days (Manolagas, 2000). After osteoclasts have eroded a particular amount of bone, they die and quickly removed by macrophages. Both osteoblasts and osteoclasts and some of osteocytes die by apoptosis. Apoptosis is controlled by two distinct signalling pathways, one initiated by death receptors and the other regulated by the Bcl-2 family of proteins. Both activate a family of proteolytic enzymes called caspases that induce the morphological changes in apoptosis by cleaving specific substrates (Xing and Boyce, 2005). Growth factors and cytokines that stimulate osteoclast and osteoblast development also influence their apoptosis.

1.4.3 Bone remodelling

Bone is constantly undergoing bone remodelling which is a complex process involving the resorption of bone on a particular surface, followed by a phase of bone formation. Resorption of bone is the task of osteoclasts while formation of new bone is the task of osteoblasts. Bone resorption and formation, however are not separate, independently regulated processes. Bone remodelling occurs in a small packet of cells called basic mulicellular units (BMUs), which turn bone over in multiple bone surfaces. Each BMU is separated chronologically and geographically from other packets of remodelling. The signal that initiates bone remodelling has not been identified but several factors have been suggested including mechanical stress. The sequence of events in

physiological remodelling are always the same, osteoclastic bone resorption, a reversal phase, followed by osteoblastic bone formation (figure 1-3).

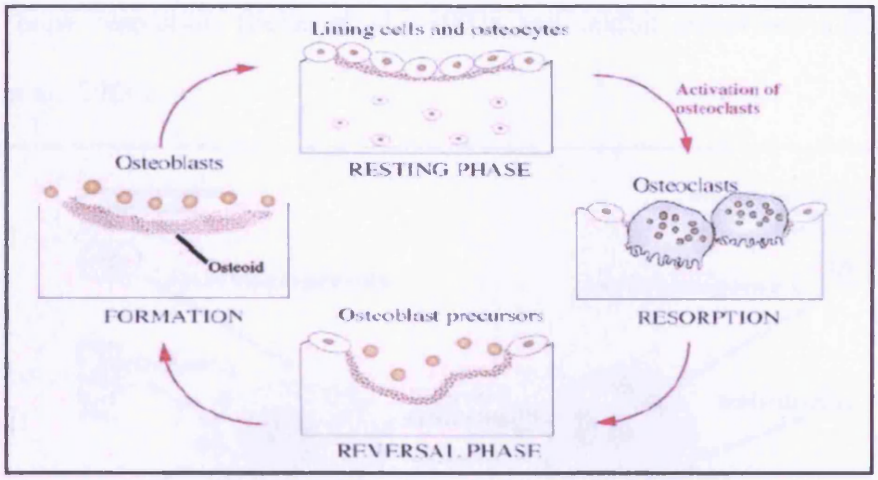

Figure 1-3: Stages of bone remodelling. Resorptive phase, reversal phase, formative phase, and resting phase. The diagram is a modification of that described by Hill, 1998.

1.4.3.1 Regulation of bone remodelling

Bone remodelling is controlled by a wide variety of systemic factors including hormones and steroids and local factors such as prostaglandins, leukotrienes, cytokines, and growth factors (Nair et al., 1996). In addition, it has been reported that the process of bone formation is also controlled at the nuclear level. Transcription factors, such as the core binding factor (Cbf)aI/Runx2 (Bergwitz et al., 2001) and Osterix (Nakashima et al., 2002) have been shown to play a role in bone metabolism. Bacteria can also affect bone remodelling by different mechanisms including modulation of bone cell functions and stimulation of the production of local factors such as cytokines. Some bacteria are able to produce osteolytic components which can cause bone resorption. An example of these osteolytic materials is the surface associated materials that can be produced by *S.*

epidermidis (Meghji et al., 1997). Bacteria such as *Pasteurella multocida* has been shown to produce PMT toxin (*Pasteurella multocida* toxin) (Lax and Chanter, 1990) which can stimulate bone resorption (Felix et al., 1992) and inhibit osteoblast differentiation (Harmey et al., 2004).

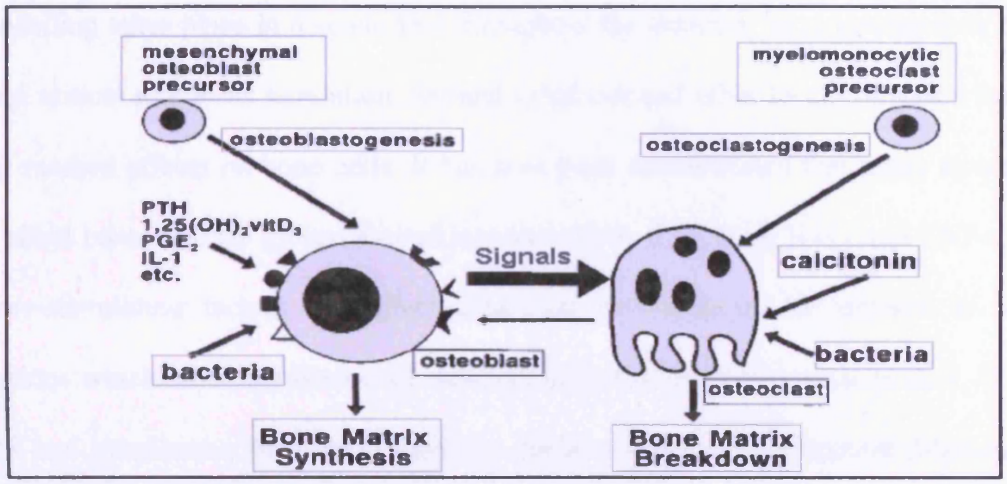

Figure 1-4: Interaction of an osteoblast and an osteoclast and mediators of bone remodelling. The diagram is a modification of that described by Nair et al, 1996.

The first stage of bone resorption involves the recruitment of osteoclast progenitors to bone. They proliferate into osteoclasts through a mechanism involving cell to cell interaction with osteoblast stromal cells (Lorenzo, 2000). Osteoblasts prepare the bone surface by removal of the unmineralised osteoid layer. This facilitates access of the osteoclasts to the underlying mineralised bone followed by osteoclast polarisation. This involves the formation of ruffled borders and clear zones, which are characteristic features of osteoclasts (Hill, 1998). Bone resorption can be initiated by local factors as well as by the interaction of osteoblasts and osteoclasts (figure 1-4). Activated osteoclasts start to resorb bone by releasing hydrogen ions and proteolytic enzymes. Osteoclasts ultimately undergo apoptosis, which is characterised by nuclear and cytoplasmic condensation, DNA fragmentation (Manolagas, 2000).

1.4.3.1.1 Cytokines and bone homeostasis

Cytokine production by host cells has been shown to provoke inflammatory reactions and local tissue damage (Gonzalez, 2000). Bone tissue is continuously remodelled by the integrated activity of osteoclasts and osteoblasts. Because bone remodelling takes place in discrete foci throughout the skeleton, local mechanisms must play a critical role in its regulation. Several cytokines and other locally released factors exert marked effects on bone cells. It has now been demonstrated that many cytokines can affect bone. A large group of cytokines such IL-1, IL-3, IL-6, IL-11 and TNF-α and colony-stimulating factors can affect osteoclast development. As opposed to these cytokines which stimulate osteoclast development other cytokines such as IL-4, IL-10, IL-18 and interferon-γ have been found to inhibit osteoclast development (Manolagas, 2000).

IL-6 has attracted particular attention because of its pathogenic role in several disease states characterised by bone remodelling and local or systemic bone resorption. Alone or in concert with other factors, IL-6 stimulates osteoclastogenesis (Manolagas, 2000). Some bacteria are able to stimulate cytokine production by host cells. For example infecting osteoblasts with *S. aureus* induces production of high levels of IL-6 and IL-12 (Bost et al., 1999). The role of cytokines in bone disease that occurs with malignancy has also been studied. In lymphoma and multiple myeloma, which are associated with increased osteoclasts activity, a variety of cytokines, including IL-6, IL-1 and TNF-α have been implicated as mediators of such effect (Daroszewska et al., 1999).

1.4.3.1.2 Nitric oxide and bone

Nitric oxide (NO) is a free radical, which is generated in host cells by nitric oxide synthase, and is involved in the regulation of many physiological processes, such as platelet aggregation and immune system regulation. It can also be generated nonenzymatically from nitrate. Nitric oxide is a highly reactive molecule, and because of this has many potential molecular targets (van't Hof and Ralston, 2001). It has become apparent that NO has important roles in bone cell function. Initially it was thought that the inhibitory effect of NO on osteoclastic bone resorption was mediated by a cyclic guanosine monophosphate (cGMP) independent mechanism, but recent evidence suggests that a cGMP dependent pathway is also involved. NO has a biphasic effects on osteoclast bone resorption. Low concentrations of NO have been shown to induce IL-1 mediated bone resorption (Ralston and Grabowski, 1996). Although it has been suggested that NO is required for osteoclast function, examination of bones from animals with NO deficiency has shown no major defect in bone resorption. Osteoporosis due to inflammation has been suggested to be mediated by activation of NO (Armour et al., 1999). High concentrations of NO can inhibit osteoclast formation and activity and consequently bone resorption. Similar biphasic effects can be seen in osteoblasts where low concentrations of NO can stimulate osteoblast growth and cytokine production while high concentrations inhibit osteoblast growth and differentiation (Ralston and Grabowski, 1996; Lin et al., 2003). Other researches have shown that low concentrations of NO have little effect on osteoblasts. It has been shown that inhibitors of NO can reduce the severity of arthritis in animals (McCartney-Francis et al., 1993). This suggests that these inhibitors may have an important clinical use as therapeutic agents (van't Hof and Ralston, 2001).

1.4.3.1.3 Systemic hormones and bone

Two main hormones of the calcium homeostatic system, namely parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 are portent stimulators of osteoclast formation. These hormones regulate calcium absorption and excretion from the intestine and kidney respectively. PTH and 1,25-dihydroxyvitamin D_3 stimulate the production of IL-6 and IL-11 by osteoblasts (Hill, 1998). On the other hand calcitonin, a bone-regulating hormone, inhibits osteoclast development and activity and promotes osteoblast survival and delays apoptosis (Selander et al., 1996). Several other hormones such as estrogen and glucocorticoids can regulate the development of osteoclasts and osteoblasts by induction of cytokines (Manolagas, 2000).

1.4.3.1.4 The RANK/RANKL/OPG system

An osteoblast activating factor (OAF) has been shown to be produced by osteoblastic stromal cells in response to a variety of stimuli. This factor has been recently identified as RANKL (receptor activator of NF-$_K$B ligand), also known as OPGL, which is a membrane protein expressed on osteoblastic stromal cells and belongs to the tumor necrosis factor family (Yasuda et al., 1998). Osteoclasts express the receptor for RANKL. This has been named RANK (receptor activator of NF-$_K$B) (Nakagawa et al., 1998). The interaction between RANKL and RANK has been shown to be essential in osteoclast development. Osteoprotegerin (OPG), also known as osteoclast inhibitor factor (OCIF) can inhibit osteoclastogenesis by interacting with RANKL and preventing its binding to the RANK receptor. OPG is produced by osteoblastic stromal cells. Although the role of RANK/RANKL/OPG system in bone diseases has not been well studied, it has been

suggested that abnormalities in this system may be involved in skeletal disorders (Gonzalez, 2000).

1.5 Epithelial cells

1.5.1 Epithelium

Epithelium is one of the primary tissues. It consists of cells that are closely opposed to one another and occur in various morphological types. The epithelial tissues are divided into two major types, the covering and lining epithelial membranes and the glandular epithelium.

1.5.2 Skin wound healing

Skin wound healing is a dynamic process in which a temporary fibrin clot is formed and provides a transient substrate for platelets that produce cytokines, growth factors and extracellular matrices. In normal skin, keratinocytes are joined together by cell junctions, which include desmosomes at cell-cell contacts and hemidesmosomes at cell-substrate contacts. Desmosomes are considered one of the most abundant types of cell to cell attachment in the skin epidermis layer (Kitajima, 2002). Hemidesmosomes are specialised junctions between host cells and the extracellular matrix which stabilise the epithelial cells adhesion to the underlying basement membrane. Skin epithelial histology can be seen in figure 1-5.

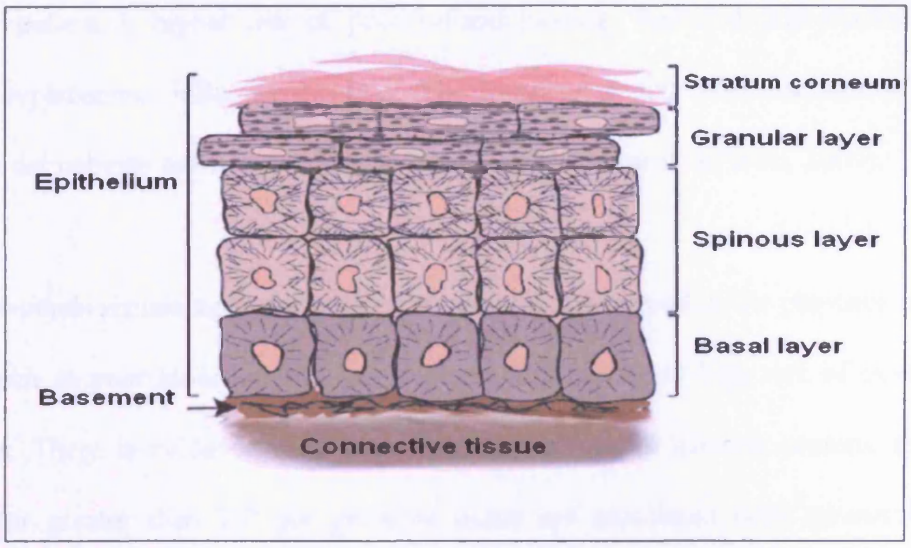

Figure 1-5: Diagrammatic representation of skin epithelium histology. The diagram is a modification of that described by Alonso and Fuchs (Alonso and Fuchs, 2003).

In healing of skin epithelium, cells undergo hemidesmosome dissociation to release cell-substrate contacts and allow migration of cells. Migration of keratinocytes also involves dissolution of most desmosomes, retraction of intracellular filaments and formation of peripheral actin filaments and focal contacts (Santoro and Gaudino, 2005). Keratinocytes then proliferate to form a dense hyperproliferative epithelium. This proliferation involves interaction with growth factors (GFs), integrins and metalloproteases (MMPs). Three growth factors have been shown to play a central role in the proliferation process. These include epidermal growth factor (EGF), transforming growth factor- α (TGF-α) and keratinocytes growth factor (KGF) (Werner and Grose, 2003).

1.5.3 Factors that affect wound healing

Many factors have been suggested to play a role in impaired wound healing. These include hydration, tissue oxygen and perfusion, nutrition, infection and molecular abnormalities at the wound site. The physiological changes that occur with ageing place

the older patient at higher risk of poor wound healing. Reduced skin elasticity and collagen replacement influence healing. The immune system also declines with age making older patients more susceptible to infection (Gosain and DiPietro, 2004).

Chronic wounds remain open for prolonged periods of time and in the presence of other factors such as poor blood supply and hypoxia they can be at high risk of developing infections. There is evidence that, regardless of the type of bacteria present, numbers equal to/or greater than 10^6 per gram of tissue are associated with serious healing impairment (Falanga, 2004). There has been increasing interest in the possible presence of biofilms in chronic wounds and their role in wound healing impairment. Ischemia can lead to tissue necrosis and often result in wounds that are more easily compromised by infection. In contrast to ischemia, hypoxia (low levels of oxygen) can stimulate host cell proliferation and enhance the synthesis of growth factors (Gottrup, 2004). However, there is also evidence that exposure to hyperbaric oxygen can enhance wound healing (Broussard, 2004). It is possible that initial hypoxia works as a stimulus but prolonged hypoxia can lead to impaired wound healing.

1.6 Inhibitors used to examine bacterial interactions with host cells

One of the ways to investigate the internalisation mechanism of bacteria by host cells is by the use of different inhibitors that can interfere with bacterial or host cell function or structure. There are a number of inhibitors of the host cell cytoskeleton and receptor recycling which can be used to study the uptake of bacteria by host cells and these can be divided into three main categories according to their target or action. The first group of inhibitors are the ones that affect microtubules which are part of the cytoskeleton of the host cell. These are colchicine, nocodazole and taxol. Colchicine and

nocodazole cause depolymerisation of microtubules, while taxol stabilises microtubules. The second group are those that affect host cell microfilaments (actin filaments). These include cytochalasin D, latrunculin B and jasplakinolide. Both cytochalasin D and latrunculin B cause depolymerisation of microfilaments while jasplakinolide stabilises polymerised actin filaments. Latrunculin B is considered a potent actin depolymeriser compared to cytochalasin D. The third group of inhibitors are those which affect clathrin coated vesicle formation and receptor recycling such as monodansylcadaverine, monensin and ouabain.

1.6.1 Inhibitors of microtubule polymerisation

1.6.1.1 Colchicine

Colchicine is a water-soluble alkaloid that blocks or suppresses host cell division by inhibiting the development of spindles and nuclei division during mitosis. Normally, the cell would use its spindle fibres to line up its chromosomes, make a copy of them and then divide into two daughter cells having the same number of chromosomes. It disrupts the cell's microtubules by binding to tubulin and preventing its polymerisation (Graening and Schmalz, 2004). It also induces apoptosis in several normal and tumor cell lines (Suzuki et al., 1998). It can inhibit adhesion of neutrophils and granulocytes to the epidermis (Modschiedler et al., 2000). Colchicine has proven to have a narrow range of effectiveness as a chemotherapeutic agent. It is used in treating gout and occasionally in veterinary medicine to treat cancers. It is also used as an antimitotic agent in cancer research involving cell cultures. It can inhibit the uptake of bacteria by preventing polymerisation of host cell microtubules (Bower et al., 2005).

1.6.1.2 Nocodazole

This inhibitor interferes with the cell cycle at G/M phase by destabilising cellular microtubules. It is an antimitotic agent that disrupts host cell microtubules by binding to tubulin and preventing the formation of the two disulfide linkages, thus inhibiting microtubule dynamics, and disrupting mitosis (Vasquez et al., 1997). It has been reported that it depolymerises axonal microtubules and consequently inhibits axoplasmic transport (Mejillano et al., 1996). The uses of nocodazole have included knocking out microtubule structures from cells cultured *in vitro* in order to arrest them at mitosis (Mejillano et al., 1996). It can prevent phosphorylation of the T cell antigen receptor inhibiting its activity (Huby et al., 1998) and induces apoptosis in several normal and tumour cells (Zhang et al., 2002). By interfering with microtubule dynamics it can affect the internalisation of bacteria which may utilise such structures during uptake by host cells (Kuhn, 1998).

1.6.1.3 Taxol

Taxol (paclitaxel) is a natural anti-tumor agent obtained via a semi synthetic process. It binds to the N-terminal region of β-tubulin and promotes the formation of highly stable microtubules that resist depolymerisation, thus preventing normal cell division by arresting the cell cycle at G2/M phase (Parekh and Simpkins, 1997). In addition it is highly lipophilic and insoluble in water. It has been demonstrated that taxol can activate the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK). The JNK/SAPK signalling pathway responds to various stress-related stimuli and is involved in initiation of apoptosis in many cell types (Wang et al., 1998). Taxol induces apoptosis of the host cell through a c-Jun N-terminal kinase -dependent pathway followed by a JNK-independent pathway, perhaps related to the activation of protein kinase A or raf-1

kinase, that results in phosphorylation of Bcl-2 (Wang et al., 1998). Taxol has been used to assess the role of stabilising host cell microtubules in the process of internalisation of many bacteria by host cells (Oelschlaeger and Tall, 1997).

1.6.2 Inhibitors of host cell microfilament polymerisation

1.6.2.1 Cytochalasin D

Cytochalasin D is a cell permeable fungal toxin, and potent inhibitor of actin polymerisation (Loty et al., 1995). It disrupts actin microfilaments and activates p53-dependent pathways causing arrest of the cell cycle at the G1-S transition phase (Rubtsova et al., 1998). It can inhibit smooth muscle contraction (Youn et al., 1998) and insulin-stimulated transport of glucose (Tsakiridis et al., 1994). Microfilament dependent internalisation is a characteristic feature of many bacteria (Hudson et al., 1995; Menzies and Kourteva, 1998; Jevon et al., 1999; Marouni and Sela, 2004) however the role of actin in endocytosis in mammalian cells remains poorly understood. Cytochalasin D can destabilise actin filaments, inhibit receptor mediated and fluid-phase endocytosis at the apical surface of polarized kidney cell but has no effect on endocytosis at the basolateral surface (Jackman et al., 1994). Although it has an inhibitory effect on the internalisation of *S. aureus* by osteoblasts (Jevon et al., 1999), cytochalasin D can increase the internalisation of other organisms. For example it can increase the internalisation of *Actinobacillus actinomycetemcomitans* by human oral epithelial cells (Brissette and Fives-Taylor, 1999).

1.6.2.2 Latrunculin B

Latrunculin B is a structurally unique marine toxin that inhibits actin polymerisation *in vitro* and disrupts microfilament organisation as well as microfilament related processes. It is 10 to 100 fold more potent than cytochalasin D. Relative to the cytochalasins, it is effective at lower concentrations and acts much more rapidly (Wakatsuki et al., 2001). Latrunculin B is produced by various species of sponge such as *Negombata magnifica* and *Latrunculia magnifica* from the Red Sea (Gillor et al., 2000). Latrunculin B forms a 1:1 complex with actin monomers and thereby inhibits polymerisation therefore it has been used to examin actin function in a variety of systems including inhibiting actin dependent internalisation of bacteria (Lamaze et al., 1997).

1.6.2.3 Jasplakinolide

Jasplakinolide is a macropeptide isolated from the marine sponge *Jaspis johanstoni* and works as a potent actin stabiliser. Unlike other known actin stabilisers such as the phalloidins and vitrotoxins, jasplakinolide appears to be a somewhat potent cell actin stabiliser and therefore can potentially be used to manipulate actin polymerisation in live cells. It has fungicidal and growth inhibiting activities and can induce programmed cell death in several cell lines (Odaka et al., 2000). It is useful for investigating cell processes mediated by actin rearrangement, including cell adhesion and internalisation.

1.6.3 Inhibitors of clathrin coated vesicle formation and receptor recycling

1.6.3.1 Monodansylcadaverine (MDC)

MDC inhibits transglutaminase which interferes with receptor recycling. In 1994, Rikihisa *et al.*, showed that the use of monodansylcadaverine inhibits infection and internalisation of *Ehrlichia risticii* by macrophages (Rikihisa et al., 1994). It is also considered an inhibitor of alpha-2 macroglobulin, epidermal growth factor, and T_3 uptake in fibroblasts. It has toxic effects at a low dose on rat neuronal cultures (Dickson et al., 1981). It can be used to test the role of receptor recycling in host cells in the uptake of bacteria.

1.6.3.2 Ouabain

Ouabain is a cardiac glycoside that selectively inhibits sodium-potassium ATPases by binding to the alpha subunit near the amino terminal and to the H5-H6 region, which may block the active efflux of sodium and reuptake of potassium (Manunta and Ferrandi, 2004). The way that ouabain may inhibit the internalisation of some bacteria is by interfering with the process of clathrin coated vesicle formation through its interaction with clathrin adapter proteins (Oelschlaeger and Tall, 1997). Clathrin coated vesicles are lipid vesicles surrounded by a closed network of hexagonal and pentagonal facets. The basic subunit of clathrin coats is a single protein called clathrin. These vesicles help in transport of materials across the cytoplasmic membrane (Mashl and Bruinsma, 1998).

1.6.3.3 Monensin

Monensin is a sodium ionophore that blocks glycoprotein secretion and may induce catecholamine secretion from chromaffin cells. It can downregulate receptor recycling by inhibiting endosome acidification (Carpentier et al., 1984). It can inhibit internalisation of bacteria which use receptor recycling and endosome acidification as part of their internalisation mechanism. Monensin has an inhibitory effect on the internalisation of *S. aureus* by osteoblasts (Jevon et al., 1999). It has been shown that monensin can block the uptake of *Campylobacter jejuni* by human embryonic intestinal cells (Biswas et al., 2000). Monensin has been shown to induce apoptosis in tumor cells (Park et al., 2002).

1.6.4 Inhibition of bacterial de novo protein synthesis

1.6.4.1Chloramphenicol

Chloramphenicol was isolated from *Streptomyces venezuelae* in 1947, but it is now available synthetically for therapeutic use as chloramphenicol, chloramphenicol palmitate, or chloramphenicol sodium succinate. Chloramphenicol is usually bacteriostatic but may be bactericidal at high concentrations or against more susceptible organisms such as *Haemophilus influenzae*. It binds to the 50S subunit of bacterial ribosomes, and inhibits peptide bond formation. Chloramphenicol also inhibits mitochondrial protein synthesis in both bacterial and mammalian cells via its effects on the 70S ribosome at these sites. Protein synthesis of rapidly proliferating cells may be affected, especially mammalian erythrocytes. It has been widely used to investigate the

effect of de novo protein synthesis in the process of internalisation of many bacteria by host cells (Oelschlaeger and Tall, 1997; Marouni and Sela, 2004).

1.6.5 Inhibition of tyrosine protein kinase

1.6.5.1Genistein

Genistein is a specific inhibitor of tyrosine protein kinases. It is a potent inhibitor of the mammalian facilitative hexose transporter GLUT1 (Vera et al., 1996). In human HL-60 cells, which express GLUT1, inhibition of transport of dehydroascrobic acid, deoxyglucose, and methylglucose in a dose-dependent manner was observed. It has been used as a suppressive drug against cancer (Ouchi et al., 2005) and to examine the effect of tyrosine protein kinases in internalisation of a variety of bacteria by host cells, for example internalisation of *S. aureus* by osteoblasts (Hudson et al., 1995; Ellington et al., 1999). A summary of actions of the inhibitors used in the project can be seen in the following table.

Table 1-1: Inhibitors and their functions

Inhibitor	Action
Colchicine	Inhibits polymerisation of microtubules and endocytic vesicle transport
Nocodazole	Inhibits microtubule polymerisation and consequently inhibits axoplasmic transport
Taxol	Stabilises microtubules by binding to beta-tubulin
Cytochalasin D	Inhibits polymerisation of microfilaments
Latrunculin B	Inhibits microfilament depolymerisation
Jasplakinolide	Potent actin stabiliser
Mondodansylcadaverine	Inhibits transglutaminase and affect receptor recycling
Monensin	Inhibits endosome acidification and can affect receptor recycling
Ouabain	Arrests clathrin coated pit formation by inhibiting interactions between clathrin and adapter proteins
Genistein	Inhibitor of tyrosine protein kinases; competitive inhibitor of ATP in other protein kinase reactions.
Chloramphenicol	Broad spectrum antibiotic inhibits bacterial protein synthesis

1.7 Adhesion of *S. epidermidis* to surgical sutures

1.7.1 Sutures

Sutures are the most commonly used implantable devices due to their usage in daily surgical procedures. They are used to achieve wound closure when ever tissue separation has occurred due to incision or injury. The older methods of wound closure included the use of natural materials such as flax, silk and cotton. With the development of synthetic polymers and fibres, synthetic sutures were introduced. Other techniques for wound closure have been developed in recent years including clips, staples and tissue adhesives (Howell et al., 1995). These materials including sutures when used in the body can trigger an inflammatory reaction and affect wound healing (Gabrielli et al., 2001). For this reason, it is essential to consider which measures are the most useful for reducing the risk of post-operative complications such as tissue reactivity, dehiscence and infection. Some of the features of good suture materials should be that they are easy to handle, inexpensive, have a good tensile strength and induce minimal tissue reactivity.

1.7.1.1 Types of sutures

Sutures can be classified as being natural or synthetic, absorbable or non-absorbable, monofilament or multifilament.

1.7.1.1.1 Natural and synthetic sutures

Natural sutures are made of natural materials such as catgut. They have been reported to induce tissue reactions more often than synthetic sutures (Gabrielli et al., 2001). An example of a natural suture is the surgical gut or catgut which was the first absorbable suture material available. It is formed by twisting the collagen of the intestine

of sheep or cows. Three forms of gut sutures are available, plain, chromic and fast absorbing. The plain gut suture elicits a marked inflammatory reaction in the tissue. The chromic gut is a plain gut treated with chromium salts to slow its absorption and decrease tissue reactivity. Synthetic sutures such as prolene and vicryl started to replace the natural sutures due to their enhanced properties in handling and biocompatibility. Horse hair has also been used to close surgical incisions (Kravetz, 2003).

1.7.1.1.2 Absorbable and non-absorbable sutures

Absorbable sutures are made of degradable material and can be left buried within the tissue where they are degraded by host enzymes. They loose their tensile strength within 60 days after placement. Examples of absorbable sutures include vicryl and polydioxanone (PDS). Non-absorbable sutures are defined by their resistance to degradation by host tissue.

1.7.1.1.3 Monofilament and multifilament sutures

Sutures can be subclassified further to monofilament and multifilament. A monofilament suture is made of a single strand. It has been suggested that the monofilament nature of some sutures decreases the risk of wound infection (Molea et al., 2000). A multifilament suture consists of several filaments twisted or braided together, this gives good handling and tying qualities.

1.7.2 Sutures and infection

Although surgical sutures are known to increase the risk of the development of wound infections, few studies have been conducted to examine this area (Mehta et al.,

1996). The ability of bacteria to bind to biomaterials is considered a key step in the development of material-related infections. When a foreign body is inserted into the human body a number of reactions occur to expel the material. Bacterial colonisation of biomaterials does not always lead to infection. Bacteria attached to suture materials can form a biofilm in which they will be protected from host defence factors and this accounts for their long persistence on the surface until they are removed with the sutures (Otten et al., 2005). Many factors have been reported to affect bacterial adhesion to sutures these include the physical and chemical properties of the suture material. The surface configuration of sutures has been suggested to contribute more to their susceptibility to be colonised by bacteria than the surface finish (Chu and Williams, 1984).

Contamination of sutures by bacteria can occur during manipulation, insertion or even through haematogenous spread of bacteria. Removal of sutures can also introduce some bacteria into tissues. The situation is more complicated in the case of intraoral sutures where asepsis is difficult to achieve. The degree of infection has been correlated to the adherence properties of bacteria to sutures (Katz et al., 1981). Some studies have advised the use of tissue adhesives as alternatives to sutures as they have a lower infection rate (Coulthard et al., 2004). However significantly higher incidences of superficial wound infections have been found following surgical treatment for hip fractures when wound closure is performed using metallic staples compared to subcuticular vicryl suture (Shetty et al., 2004).

1.8 Aims and objectives of the study

The aims of the study involve the investigations of the possibility that *S. epidermidis* may be internalised by osteoblasts and epithelial cells, and if so, to examine the internalisation capacity of different *S. epidermidis* strains, and to gain further understanding of the mechanism of internalisation of this organism. The adhesion of *S. epidermidis* to different surgical suture materials will also be investigated. The following areas will be covered:

1- The role of host cell cytosketon including microtubules and microfilaments in the process of internalisation of *S. epidermidis*.

2- The role of host cell receptors recycling and signal transduction in the process of internalisation.

3- The effect of bacterial de novo protein synthesis on the internalisation of *S. epidermidis* by host cells.

4- The consequences of the internalisation of *S. epidermidis* by host cells including intercellular replication, cytokine production and apoptosis.

5- The role of some known *S. epidermidis* virulence factors in the internalisation of this bacterium by host cells.

6- The interaction of *S. epidermidis* with different sutures materials and the role of different virulence factors in this interaction.

Chapter 2

Chapter 2

Materials and methods

2.1 Osteoblast and epithelial cell culture

The human osteoblastic cell line MG63 and the human laryngeal cancer cell lines Hep2 were routinely cultured in Dulbecco's modified Eagle's medium (DMEM), containing 25 mM HEPES (4-2-hydroxyethyl-1-piperazineethanesulfonic acid) and supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich Ltd, United Kingdom), 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml of streptomycin (Sigma, UK). Cells were cultured in 75 cm^3 flasks (BDH, UK) in 20 ml of the culture medium at 37°C in an atmosphere of 95% air and 5% CO_2.

2.1.1 Cell counting

Cell counting was performed using a hemocyotmeter. Cells were diluted 1:10 then 10 μl was transferred to the hemocytometer and the cells in the four large squares (1 mm^2 each) contained within squares at the corner and the middle were counted. The number of cells was divided by 0.1 mm (the depth of the chamber) then by the total surface area of squares used for counting to obtain the number of cells per cubed millimetre. To calculate the number of cells per 1 ml the number then multiplied by 1000 (there are 1000 mm-cubed in 1 ml).

2.1.2 Cell freezing

Osteoblasts or epithelial cells were washed with PBS and harvested using 0.25% trypsin (Sigma, UK). Cells were then resuspended in DMEM (approximately 5 x 10^8

cells) containing 10% dimethyl sulfoxide (Me_2SO). Cells were then transferred into a freezing container containing isopropyl alcohol and stored at -70°C for short term storage. For long term storage cells were stored first at -70°C overnight then transferred to liquid nitrogen.

2.1.3 Cell fixation

Cells on cover slips were treated with 4% paraformaldehyde solution (the solution was obtained from the electron microscopy department, Eastman Dental Institute, UK) for 10 minutes at room temperature then washed three times with PBS.

2.2 Staining

2.2.1 Trypan blue staining

Trypan Blue was diluted to 0.8 mM in PBS and osteoblasts were incubated with the dye for 1 minute. Dead cells stain blue, while live cells exclude the trypan blue. This procedure cannot distinguish between necrotic and apoptotic cells.

2.2.2 Phalloidin-rhodamine staining

Osteoblasts grown on cover slips (22 x 22 mm, BDH, UK) in 6-well plates (BDH, UK) were washed twice with prewarmed (37°C) PBS, then fixed with 4% formaldehyde solution in PBS for 10 minutes at room temperature and washed three times with PBS. Cells were permeablised by incubation with 0.1 % Triton X-100 for 3 minutes. Osteoblasts were then washed three times with PBS. F-actin staining was performed by adding 1 U/ml of rhodamine-conjugated phalloidin. Cells were incubated for 20 minutes in the dark at room temperature and then washed three times with PBS.

2.3 Bacterial strains and growth

2.3.1 Bacterial strains

2.3.1.1 *S. epidermidis*

The *S. epidermidis* strains used in this thesis were NCTC11047 and NCTC11964 (from the National Collection of Type Cultures, Central Public Health Laboratory Service, London, UK). *S. epidermidis* strain 19 a fibrinogen binding isolate from a patient with peritonitis was obtained from Dr Jan-Ingmar Flock, the Karolinska Institute, Sweden. *S. epidermidis* strain HB and its isogenic mutants deficient in SdrG were obtained from Professor Tim Foster, Trinity College, Dublin, Ireland. *S. epidermidis* isogenic mutants, Embp3349 and Embp284335 were produced by Dr. Rachel Williams in our laboratory. Strain 9 and its isogenic mutants 2J24 (*gehC*::*ermC*), and KIC82 (*gehD*::*ermC*) were a gift from Professor Keith Holland, School of Biochemistry and Molecular Biology, University of Leeds, UK. Strain O-47 and its isogenic mutants deficient in PIA or AtlE were obtained from Professor Paul Fey, Nebraska Medical Center, University of Nebraska, Nebraska, USA. *S. epidermidis* strain RP62A is a biofilm producing strain (table 2-1).

2.3.1.2 *S. aureus*

S. aureus strain NCTC6571 (NCTC, Central Public Health Laboratory Service, London, UK) is a laboratory strain commonly used as a control in the tube coagulase test and for antibiotic sensitivity testing. *S. aureus* strain LS-1 is an isolate from a swollen joint of a spontaneously arthritic NZB/W mouse and was obtained from Dr. Andrej Tarkowski, Department of Rheumatology, University of Goteborg, Sweden (table 2-1).

Table 2-1: Staphylococcal strains used in the study

Strain	Relevant characteristics
S. epidermidis	
NCTC11047	A type strain of *S. epidermidis*
NCTC11964	A type strain of *S. epidermidis* that produces enterotoxin C
19	A fibrinogen binding isolate from a patient with peritonitis
HB	An isolate from a patient with osteomyelitis
HB-SdrG -	Mutant of *S. epidermidis* HB disrupted in the gene for SdrG
HB-embp3349	An isogenic mutant of HB with a disruption at the start of the gene for Embp
HB-embp284335	An isogenic mutant of HB with a disruption in the 3' prime end of the gene coding for Embp
O- 47	A biofilm-positive clinical isolate
O-47 PIA -	A Tn*917* insertional mutant of O-47 that is deficient in PIA production because of an interruption in the *ica* (*icaA*) locus
O-47 AtlE -	A Tn*917* insertional mutant of O-47 that is deficient in AtlE production because of insertion into the *atlE* gene
9	Wild-type isolated from volar forearm
2J24	Isogenic mutant of *S. epidermidis* 9; *gehD*+ *gehC*: :*ermC*
KIC82	Isogenic mutant of *S. epidermidis* 9; *gehC*+ *gehD*: :*ermC*
RP62A	A biofilm-forming strain
S. aureus	
NCTC6571	Laboratory strain
LS-1	Pathogenic strain producing TSST-1, β-hemolysin and enterotoxins

2.3.2 Bacterial growth

Bacteria were routinely grown in brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) aerobically at 37°C with shaking.

2.3.2 Determination of bacterial numbers

Bacteria were suspended in 50 ml falcon tubes containing 10 ml of brain heart infusion (BHI) and incubated for 15-18 hours with constant shaking (200 rpm) at 37°C. Then different dilutions of the overnight bacterial culture were made in PBS. The optical density of these dilutions was measured using a spectrophotometer at wavelength of 600 nm. Serial dilutions from the overnight cultures were plated on blood agar plates containing 5% horse blood. The relation of the optical density and numbers of colony forming units per ml was analyzed and a standard curve was obtained. Bacterial numbers in subsequent overnight cultures were determined according to this curve.

2.3.3 Bacterial staining and labeling

2.3.3.1 Gram staining

A monolayer of bacteria was smeared on a glass slide, left to dry then passed 3 times over a flame. Crystal violet stain was added to the smeared layer for 30 seconds followed by gentle washing under running water. Iodine solution was then added for 30 seconds and rinsed with flowing water. Cells were decolorized using acetone for 5 seconds then washed with water. Safranin was added to the slide for 30 seconds and then removed by washing with water. Slides were air dried and examined using a light microscope.

2.3.3.2 Labeling of *S. epidermidis* with an anti-lipoteichoic acid antibody

Lipoteichoic acid (LTA) is a major proinflammatory protein present within the cell wall of most Gram-positive bacteria. Internalisation assays were performed as described in section 2.5.2. Osteoblasts were fixed with 4% paraformaldehyde for 10 minutes then washed three times with PBS. Osteoblasts were permeabilised by incubation with 0.1% Triton X-100 for 3 minutes then washed 3 times with PBS. 5% goat serum (Sigma, UK) in PBS was added to the osteoblasts to block non-specific binding sites. Osteoblasts were then washed 3 times with PBS. An antibody to lipoteichoic acid (Autogen-Bioclear, UK) was added to the cell at a concentration of 0.1μg/ml and incubated for 1 hour before the osteoblasts were washed 3 times with PBS. Fluorescein-5-isothiocyanate (FITC) labeled antibody to mouse IgG (Sigma, UK) was diluted 1:100 and incubated with osteoblasts in the dark for one hour. Osteoblasts were stained with rhodamine-conjugated phalloidin as described in section 2.2.2. Osteoblasts and *S. epidermidis* were visualised using the confocal microscope as described in section 2.6.

2.5 Bacterial association and internalisation assays

2.5.1 Association of *S. epidermidis* with host cells

The osteoblasts or epithelial cells were routinely cultured in Dulbecco's modified Eagle's solution (DMEM) supplemented with 10% fetal calf serum (FCS) and 2mM L-glutamine and containing 25 mM HEPES, 100 U/ml penicillin (Gibco, Paisley, United Kingdom), streptomycin (100 μg/ml; Gibbco). Cells were seeded at 50,000 cells per well into 24-well tissue culture plates in one millilitre of growth medium, and cultured until 80 % confluent. One day before the addition of bacteria, the cells were washed twice with

one millilitre of phosphate buffered saline (PBS), resuspended into 1ml of the antibiotic free DMEM containing 10% FCS and incubated at 37°C overnight in an atmosphere of 95% air and 5% CO_2. the numbers of bacteria to be added to the host cells were determined as described in section 2.3.2. A bacterial inoculum containing approximately 10^7 colony forming units suspended in DMEM was added to each well (multiplicity of infection of 200:1), and the plates were incubated at 37°C in a 5% CO_2 and 95% air atmosphere for 2 hours. Non-adherent bacteria were removed by washing eukaryotic cells three times with PBS. Eukaryotic cells were then lysed with 1 ml of 0.1% Triton X-100 in PBS. The adherent bacteria plus intracellular bacteria which represent the associated bacteria with host cells were determined in colony forming units per ml by 10-fold serial dilutions and plating on blood agar plates containing 5% horse blood. The number of *S. epidermidis* associated with host cells represents the number of adherent bacteria in addition to intracellular bacteria.

2.5.2 Internalisation of *S. epidermidis* by host cells

The initial steps described in section 2.5.1 for determining the number of *S. epidermidis* associated with host cells were followed. However after two hours of co-culture of the bacteria with host cells the cultures were washed three times with PBS to remove non-adherent bacteria and 1ml of growth medium containing 10% FCS and gentamicin (100μg/ml) was added to each well and incubated for an additional two hours to kill the attached (non-internalised) bacteria. Cells were then washed three times using PBS and lysed using 1ml of 0.1 % Triton X-100 in PBS. The content of each well was transferred to microcentrifuge tubes and serial dilutions were carried out in a 96-well plate. To quantify the number of internalised bacteria, these serial dilutions were plated in

triplicate on blood agar plates containing 5% horse blood followed by incubation at 37°C overnight to allow enumeration.

2.5.2.1 Detection of cytokine production by osteoblasts after internalisation of bacteria

Cytokines production by osteoblasts was examined in the presence of either *S. epidermidis* strain 19 or strain NCTC11964. *S. epidermidis* strains 19 and NCTC 11964 were incubated with a confluent monolayer of MG63 cells at a multiplicity of infection of 200 bacteria to one osteoblast. Wells containing MG63 cells and where no bacteria were added were used as a control. After incubation with gentamicin for 2, 12, 24 and 48 hours, supernatants were collected from each well in 1.5 ml microcentrifuge tubes, centrifuged at 5,000 xg for ten minutes then stored at - 70°C.

The harvested supernatants were tested for the levels of interleukin 6 (IL-6), IL-8, IL1- β and tumor necrosis factor α (TNF-α) using enzyme linked immunosorbent assays (ELISA). These are sensitive enzyme immunoassays that can specifically detect the concentration of soluble cytokine and chemokine proteins. The procedure involves the use of highly-purified anti-cytokine antibodies (capture antibodies) which are noncovalently adsorbed primarily as a result of hydrophobic interactions onto plastic microwell plates. After plate washings, the immobilised antibodies serve to specifically capture soluble cytokine proteins present in samples which are applied to the plate. After washing away unbound material, the captured cytokine proteins are detected using a biotin-conjugated anti-cytokine antibody (detection antibodies) followed by an enzyme-labeled avidin (avidin-horse radish peroxidase) or streptavidin stage. Following the

addition of a chromogenic substrate, the level of coloured product generated by the bound, enzyme-linked detection reagent can be conveniently measured spectrophotometrically using an ELISA-plate reader at an appropriate optical density.

2.5.2.1.1 IL-6 detection using ELISA

96-well flat-bottomed plates (NUNC Immunoplate Maxisorb) were coated with 1μg/ml of anti-human IL-6 monoclonal antibody (an affinity-purified goat anti-IL-6, National Institute of Biological Standards (NIBSC), Potters Bar, UK) diluted in phosphate buffered saline (PBS, pH 7.2). 100μl of the diluted coating antibody solution was added to each well. The plates were wrapped in foil and incubated overnight at 4°C. After overnight incubation the coated plates were washed three times with wash buffer (pH 7.2) consisting of 0.5M NaCl, 2.5 mM $NaH_2PO_4.2H_2O$, 7.5mM Na_2HPO_4 and 0.1% Tween-20.

Standards of known concentration of recombinant human IL- 6 (NIBSC, UK) were diluted with wash buffer to give a range of 12 pg/ml to 6000 pg/ml. 100 μl of the diluted standard was added to the allocated well in triplicates. The harvested media from internalisation assays were diluted 1:5 or 1:10 and 100 μl of the neat and diluted samples were put into the allocated wells in the 96-well plates in triplicate. Plates were incubated for 2 hours at 37°C then washed three times with 200 μl/well wash buffer. Biotinylated anti-human IL-6 (NIBSC, UK) was diluted 1:250, then 100 μl was added to each well and incubated for one hour at 37°C. Plates were washed three times with wash buffer and 100 μl of a 1:4000 diluted avidin-horse radish peroxidase (Dako Ltd, Buckinghamshire, UK) solution was added to each well. The plates were incubated at 37°C for 15 minutes then washed three times with wash buffer. Substrate was prepared by dissolving a 10 mg

O-phenylenediamine tablet (Sigma) in 25 ml of 34.7 mM citric acid (7.98 g/L: H_2O), pH 5.0, and adding 10 µl of 30% hydrogen peroxide (Sigma). The substrate (100 µl) was added to each well and colour allowed to develop.

The reaction was terminated using 150 µl/well of 1M sulfuric acid (H_2SO_4, Sigma). The absorbance was measured at 490 nm using Dynex plate reader. The software package, Revelation was used to generate the standard curve of the absorbance versus concentration of IL-6.

2.5.1.1.2 IL-8 detection using ELISA

The ELISA to detect IL-8 in the media harvested from internalisation assays was performed following that described for IL-6. The exceptions were in the use of polyclonal sheep coating antibody to human IL-8 (NIBSC, UK) and standards (sample with known IL-8 concentration, NIBSC, UK). The biotinylated anti-IL-8 (NIBSC, UK) was diluted at 1:200 and the standard was diluted to give a range of 20 to 10000pg/ml.

2.5.1.1.3 IL-1β detection using ELISA

The ELISA to detect IL-1β in the media harvested from internalisation assays was performed following that described for IL-6. The exceptions were in the use of immuno-affinity purified goat antibody to human IL-1β (anti-human IL-1β, NIBSC, UK) and standards (sample with known IL-1β concentration, NIBSC, UK). The biotinylated anti-IL1β (NIBSC, UK) was diluted at 1:500 and the standard was diluted to give a range of 16 to 8000pg/ml.

2.5.1.1.4 TNF-α detection using ELISA

The ELISA to detect TNF-α in the media harvested from internalisation assays was performed following that described for IL-6. The exceptions were in the use of monoclonal mouse antibody to human TNF-α (NIBSC, UK) and TNF-α standards (sample with known concentration of human TNF-α, NIBSC, UK). A blocking step was introduced by adding 100 µl of 1% ovalbumin (Sigma) to each well prior to adding the standards and the harvested internalisation medium, then incubated at room temperature for one hour. The biotinylated polyclonal anti-TNF-α (NIBSC, UK) was diluted at 1:1000 and the standard was diluted to give a range of 41 to 20000pg/ml.

2.6 Confocal laser scanning microscopy

Osteoblasts were seeded onto 19 mm glass coverslips in 24-well plates. Cells were infected with *S. epidermidis* at a multiplicity of infection (MOI) of 200:1 for two hours. The monolayer of osteoblasts was washed three times with PBS and then incubated in growth medium containing 10% FCS and gentamicin (100 µg/ml) for two hours before washing three times with PBS. Cells were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS and then F-actin was stained by adding 1 U/ml of rhodamine-conjugated phalloidin and incubating cells for 20 minutes in the dark before they were washed three times with PBS. Fluorescence was monitored with a Leica confocal laser scanning microscope (Heidelberg, Germany) using 10% laser power, optimum iris, and Kalman 6 settings. Images were saved as multistack TIFF files which were manipulated using Leica and photoshop software.

2.7 Expression and purification of recombinant FnBPB[D1-D4]

2.7.1 Expression of recombinant FnBPB[D1-D4]

E. coli M607/pREP4-pQE30-rFnBPB[D1-D4], harboring a fragment of the gene for FnBPB in the pQE30 expression vector was grown overnight in Luria-Bertani broth (LB; Sigma) containing antibiotics (100 μg/ml ampicillin, 25 μg/ml kanamycin, 20 μg/ml spectinomycin and 20 μg/ml streptomycin; all from Sigma, UK) at 30°C. Overnight cultures were diluted 1:10 in 200 ml of fresh broth and incubated for a further 2 hour at 30°C. Gene expression was induced with 200 μl of 1M isopropyl-B-D-thiogalactopyranside (IPTG; BDH, UK) for 4 hours at 30°C. Cells were harvested by centrifugation at 6,000 x g for 20 minutes and the bacterial pellets stored at -20°C overnight.

2.7.2 Purification of recombinant FnBPB[D1-D4]

The bacterial pellets were thawed in ice for 15 minutes before resuspending the cells in 8 ml of lysis buffer consisting of Bacterial Protein Extraction Reagent (Pierce, Perbio Science UK Ltd, UK), 20 mM imidazole, 80 μl of 120 mM phenylmethanesulphonyl fluoride (PMSF), and 8μl of 1mM E-64, 10 mM leupeptin and 1mM pepstatin A (all obtained from Sigma). The cells in lysis buffer were incubated at 4°C for one hour with mixing. The cell lysate was transferred to microcentrifuge tubes and centrifuged at 14,000 x g at 4°C for 10 minutes to remove cell debris. The cleared supernatant was collected from each tube and pooled into a 15-ml falcon tube together with 1 ml of NI-NTA agarose (Qiagen Ltd, Crawley, UK) and incubated for one hour with mixing at 4°C. The lysate-Ni-NTA mixture was loaded into a polypropylene column of 4-ml total capacity (Qiagen), and the flow through was collected. The column was

washed with 4 ml of B-PER reagent, followed by 4 ml of PBS, both containing 20 mM imidazole. The column was then washed with 4 ml of 2 mg/ml of polymyxin B (Sigma) in PBS to remove any lipopolysaccharide. Following two further washes with 4 ml PBS, the bound protein was eluted with 4 x 0.5 ml PBS containing 250 mM imidazole. The purity of recombinant FnBPB[D1-D4] was determined by SDS-PAGE as described in section 2.8.

2.8 Gel electrophoresis

2.8.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a Mini PROTEAN II electrophoresis system (Bio-RAD). The separating gel (8cm x 7cm) was made with 0.4M Tris-HCl pH 8.8, 0.1% (w/v) SDS and 12% (w/v) acrylamide mix [29.2% (w/v) acrylamide, 0.8% (w/v) bisacrylamide; National Diagnostic, UK], 50µl of 10% ammonium persulphate (APS) and 5µl of TEMED (N,N,N',N'-tetramethylethylenediamine) per 5ml gel were added, to give 12% gels. Gels of 10% were prepared in the same way except that 10% acrylamide mix (w/v) was used. Once poured into the gel apparatus, the acrylamide was overlaid with distilled water to produce a flat surface to the gel and allowed to set for 45 minutes at room temperature.

The stacking gel was then prepared and poured on top of the separating gel. This 5% gel was made with 0.125M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 3.9% (w/v) acrylamide mix, 50µl 10% APS and 5µl TEMED were added per 5ml gel. A 10 well comb was inserted after pouring the gels to allow wells to form for sample application. The gel was left to set for 30 minutes at room temperature before the comb was removed and the gel

mounted in the electrophoresis apparatus. Samples were mixed with 5X ImmunoPrep (Pierce) reducing sample buffer and boiled at 95° C for 5 minutes prior to loading on the gel. SDS-PAGE running buffer (25mM Tris-HCl pH 8.3, 250mM glycine, 0.1% SDS) was added to the apparatus. Samples and broad range molecular mass markers (New England Biolabs, UK) were electrophoresised through the stacking gel at a constant current of 30 mA per gel. Once the samples had passed through the stacking gel the current was reduced to 15 mA per gel until the dye front reached the bottom of the gel.

2.8.2 Staining SDS-PAGE gels

After electrophoresis gels were fixed for one hour with a mixture consisting of 40% (w/v) methanol, 15% (v/v) acetic acid and 45 % distilled water. Brilliant Blue G colloidal concentrate (Sigma) was made up according to the manufactures instructions and four parts of stain were mixed with one part of methanol immediately prior to use. The gels were soaked overnight in the stain before destaining in water.

2.8.3 SDS-PAGE of recombinant FnBPB[D1-D4]

The SDS-PAGE was carried out using stacking and running gels with 5% and 12% acrylamide, respectively. The proteins were separated under constant voltage set at 100 V for one hour or until the electrophoresis was complete. Separated proteins on the gel were stained overnight with Brilliant Blue G-collodal concentrate (Sigma, UK) and destained in water. Eluted factions contained the protein fractions were pooled and dialysed (membrane tubing with molecular weight cut-off of 3,500; Spectrum, Medical International Ltd, UK) against PBS at 4°C. The PBS was changed twice daily for 5 days.

The protein concentration was determined by measuring the absorbance at 280 nm, using an absorbance reading of 0.220 corresponded to a protein concentration of 1 mg/ml.

2.9 Statistical analysis

Data were analysed using Student's t-test when normally distributed. Mann Whitney U test was used when data were skewed. A P value of less than 0.05 was considered statistically significant.

Chapter 3

Chapter 3

Internalisation of *Staphylococcus epidermidis* by osteoblasts

3.1 Introduction

Staphylococci were once considered to exist exclusively as extracellular pathogens. However, there is growing evidence showing that *S. aureus* is taken up by a range of non-phagocytic cells such as epithelial cells (Jett and Gilmore, 2002), endothelial cells (Yao et al., 1995; Menzies and Kourteva, 1998) and osteoblasts (Hudson et al., 1995; Ellington et al., 1999; Jevon et al., 1999; Reilly et al., 2000; Ahmed et al., 2001). *Staphylococcus epidermidis* is a major nosocomial pathogen and the most commonly isolated member of the coagulase negative staphylococci (Vuong and Otto, 2002). It does not usually cause pyogenic infections in noncompromised patients except in the case of native valve endocarditis (Huebner and Goldmann, 1999). Although few virulence factors have been identified in *S. epidermidis*, its infections are usually chronic in nature and difficult to treat (von Eiff et al., 2002). Persistent bone infections result in the chronic osteomyelitis associated with bone implanted devices (Ciampolini and Harding, 2000). In such cases antimicrobial therapy alone is often unsuccessful, and removal of the device is indicated (Schierholz and Beuth, 2001). *S. epidermidis* is responsible for the majority of cases of chronic osteomyelitis associated with orthopaedic implants (Ciampolini and Harding, 2000). With other coagulase negative staphylococci it is responsible for 20-50% of joint replacement infections (O'Gara and Humphreys, 2001). The phenomenon of microbial internalisation by host cells has been suggested to protect the microorganism from host humoral immunity and antibiotic treatment (Alexander and Hudson, 2001).

Recent studies have shown that *S. epidermidis* can be internalised by bovine mammary cells in a process that appears to be receptor mediated and exploits the host cell cytoskeleton and signal transduction pathways (Almeida and Oliver, 2001). It has also been reported that *S. epidermidis* can be taken up by endothelial cells (Merkel and Scofield, 2001) and can persist in pericatheter macrophages (Boelens et al., 2000). This intracellular persistence of *S. epidermidis* may play a role in the recurrence of infections caused by this organism (Boelens et al., 2000). From a clinical standpoint, it has become clear that patients can have recurrent attacks of osteomyelitis after completion of therapy even when the causative organisms cannot be isolated (Ciampolini and Harding, 2000). If *S. epidermidis* can be internalised by osteoblasts this may be relevant to the chronicity of bone infections caused by this organism.

This chapter describes the capacity of *S. epidermidis* to be internalised by bone cells, osteoblasts. Using a variety of inhibitors that act on eukaryotic cell structures or processes, we investigated the involvement of receptor-mediated endocytosis, endosome acidification, and the role of the cytoskeleton in the internalisation process.

3.2 Materials and Methods

3.2.1 Time course of internalisation of *S. epidermidis* by osteoblasts

The internalisation assay was performed as described above except that the time of co-culture was for 30, 60, 120, 150, 180, 210 or 240 minutes. Following a further two hour incubation in the presence of gentamicin (100 μg/ml) the osteoblasts were lysed with 0.1 % Triton X-100 in PBS. To quantify the number of internalised bacteria,

dilutions of the lysates were plated in triplicate on agar plates containing 5% horse blood and incubated overnight at 37°C.

3.2.2 Internalisation of *S. epidermidis* by osteoblasts in the presence of inhibitors

To examine the mechanism of internalisation of *S. epidermidis* by osteoblasts the internalisation assay was performed as described in chapter 2 section 2.5.2 but in the presence of either 10μM colchicine, 20μM nocodazole, 40μM taxol, 250μM monodansylcadaverine (MDC), 250μM ouabain, 40μM monensin, or 2μM cytochalasin D, 25 μM latrunculin B, 25 μM jasplakinolide or 250 μM genistein. The concentration of each inhibitor employed was chosen for maximal inhibitory effect without having an effect on the viability of the human cell monolayer as previously reported in the literature (Hudson et al., 1995; Jevon et al., 1999; Almeida et al., 2000). Cytochalasin D (Sigma, UK) causes microfilament depolymerisation and was used to examine the role of actin in the uptake process. The involvement of microtubules in the internalisation process was examined using inhibitors which cause microtubule depolymerisation, colchicine and nocodazole (Sigma) or which stabilised existing microtubules, taxol (Sigma). The role of coated pit formation in the uptake process was examined using MDC (Sigma). MDC inhibits transglutaminase which interferes with receptor recycling. Monensin was used to block endosome acidification which affects eukaryotic receptor recycling to the cell surface. These inhibitors were added to the osteoblasts one hour before adding the bacteria. Following the addition of bacteria the internalisation assay was performed as described in chapter 2, section 2.5.2. To establish if the inhibitors affected bacterial growth or viability, bacteria were inoculated in DMEM with 10% fetal calf serum with or without inhibitors at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for various

times and viable bacteria were enumerated by serial dilution and plating on blood agar plates. The assay was repeated in triplicate on three occasions.

3.2.2.1 Effect of cytochalasin D on the internalisation of *S. epidermidis* and *S. aureus* by osteoblasts

To examine the effect of cytochalasin D on the internalisation of *S. epidermidis* and *S. aureus*, the internalisation assay was performed as described in section 3.2.2 in the presence of 2μM cytochalasin D. Two strains of *S. epidermidis* (19 and NCTC11047) and one strain of *S. aureus* (NCTC 6571) were used in these experiments.

3.2.2.2 Effect of cytochalasin D on osteoblast and bacterial cell morphology

The effect of cytochalasin D on *S. epidermidis* cell numbers was investigated by incubating *S. epidermidis* at $1x10^8$ cells/ml with 2 μM cytochalasin D for two hours in DMEM containing 10% fetal calf serum, at 37°C in an atmosphere of 5% CO_2 and 95% air. Serial dilutions in triplicate were plated on agar plates containing 5% horse blood and incubated overnight. Bacterial colonies were counted and compared to the bacterial colonies grown from cultures where no cytochalasin D was added. Osteoblast and *S. epidermidis* morphology was examined under a light microscope after incubation with cytochalasin D.

3.2.2.3 Effect of cytochalasin D on the escape of *S. epidermidis* from osteoblasts

An internalisation assay in the presence of 2 μM cytochalasin D was performed as described in section 3.2.2. After two hours of incubation in the presence of gentamicin, cells were washed three times with PBS. Culture medium or culture medium containing 2

μM cytochalasin D was added to the wells and the cells were incubated at 37°C for a further hour after which the culture medium was collected from each well and serial dilutions were plated on blood agar plates containing 5% horse blood. This represented bacteria that had escaped from inside osteoblasts. Osteoblasts were lysed by adding 0.1% Triton X-100 to each well. Serial dilutions in triplicate were plated on agar plates containing 5% horse blood to enumerate internalised bacteria.

3.2.2.4 The role of de novo protein synthesis by *S. epidermidis* in internalisation of bacteria by osteoblasts

Because it is has been shown that de novo protein synthesis by some bacteria such as *Campylobacter jejuni* is important in internalisation (Oelschlaeger et al., 1993) by host cells , the dependency of *S. epidermidis* internalisation on de novo protein synthesis by this bacterium was investigated. At the start of the internalisation assay chloramphenicol at a concentration of 10 μg/ ml was added along with the bacteria. After incubation for 2 hours the osteoblasts were washed 3 times with DMEM and gentamicin (100 μg/ ml) was added to kill the external bacteria. The osteoblasts were lysed after 2 hours and intracellular *S. epidermidis* enumerated by serial dilution and plate counting. The effect of chloramphenicol on *S. epidermidis* viability was tested by incubating the bacteria in DMEM containing 10% fetal calf serum and 10 μg/ ml chloramphenicol. The number of bacteria recovered after 2 hours of incubation was compared to the number of bacteria in the control wells where no chloramphenicol was added to the osteoblasts.

3.2.3 Replication and intracellular survival of *S. epidermidis*

A standard internalisation assay as described in chapter 2, section 2.5.2 was performed except that after addition of the gentamicin the incubation time was varied from between 2 to 96 hours.

3.3 Results

3.3.1 Internalisation of different strains of *S. epidermidis* by osteoblasts

The standard internalisation assay described in chapter 2, section 2.5.2 was used to test the ability of different strains of *S. epidermidis* to be internalised by osteoblasts. Five different strains of *S. epidermidis* were used in this experiment (19, NCTC11047, HB, O47 and NCTC11964). The results in figure 3-1 show that *S. epidermidis* strains have different capacities to be internalised by osteoblasts. Some strains showed higher capacity to be internalised by osteoblasts. The capacity of *S. epidermidis* strain 19 to be internalised by osteoblasts was 7-fold greater than strain NCTC11047. *S. epidermidis* strain NCTC11964 was not internalised by osteoblasts. Dividing the number of internalised bacteria by the number of cultured osteoblasts showed that for *S. epidermidis* strain 19 there were three bacteria internalised by each osteoblast.

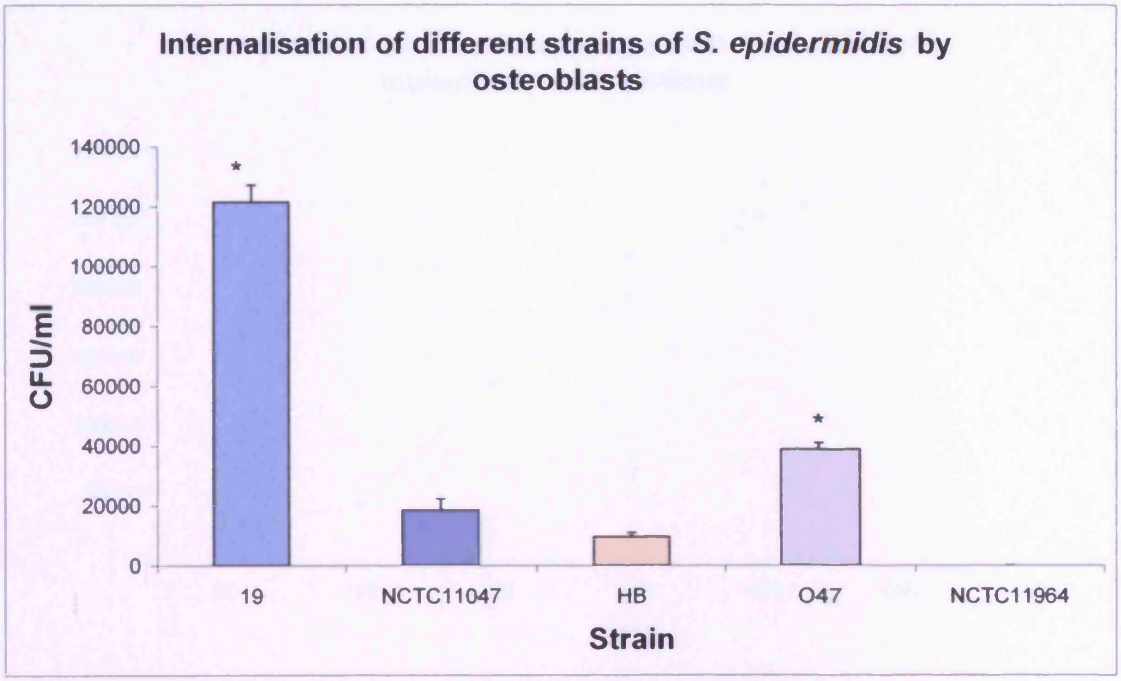

Figure 3-1: Bacterial colony-forming units per millilitre of *S. epidermidis* strains 19, NCTC11047, HB, O47 and NCTC11964 internalised by osteoblasts at a multiplicity of infection of 200 to 1. Data are the mean and standard deviation of three replicate cultures. The graph shows a representative experiment of three experiments performed on separate occasions. * P value < 0.01.

3.3.2 Effect of different multiplicity of infections on internalisation of *S. epidermidis* by osteoblasts

The effect of different MOI on the internalisation of *S. epidermidis* by osteoblasts was examined. Lowering the MOI from 200:1 to 100:1 or 50:1 decreased the number of *S. epidermidis* internalised by osteoblasts. Whereas raising the MOI from 200:1 up to 600:1 increased the number of internalised bacteria per osteoblast ($P < 0.1$). At an MOI of 200:1 three *S. epidermidis* cells were internalised per osteoblast while at a MOI of 600:1 two-fold more bacteria were internalised by osteoblasts (figure 3-2). At a MOI of 1000:1 the number of *S. epidermidis* internalised by osteoblasts dropped to 40% of that at a MOI of 600:1.

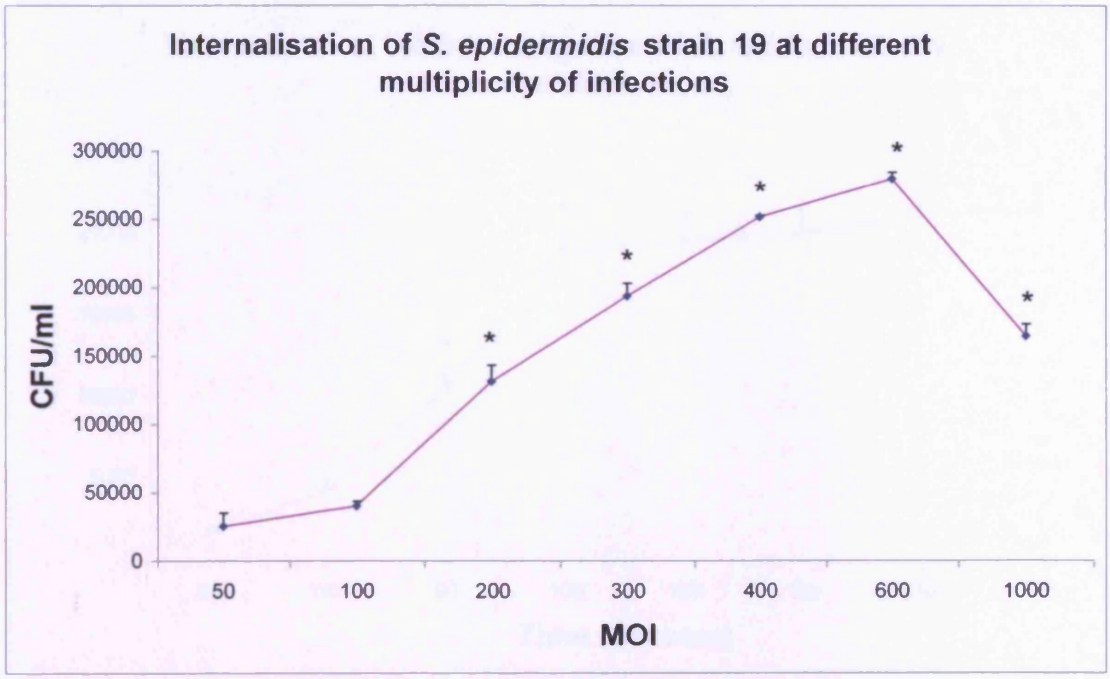

Figure 3-2: Bacterial colony-forming units per millilitre of *S. epidermidis* strain 19 internalised by osteoblasts at different multiplicity of infections. Data are the mean and standard deviation of three replicate cultures and these experiments were performed on separate occasions. * P value < 0.01.

3.3.3 Time course of the internalisation of *S. epidermidis* by osteoblasts

The kinetics of uptake of *S. epidermidis* by osteoblasts was examined. With increasing time up to 120 minutes, the numbers of *S. epidermidis* internalised by osteoblasts increased ($P < 0.01$). However the number of *S. epidermidis* internalised did not increase after 120 minutes of internalisation (figure 3-3). These experiments were repeated in triplicate on three different occasions.

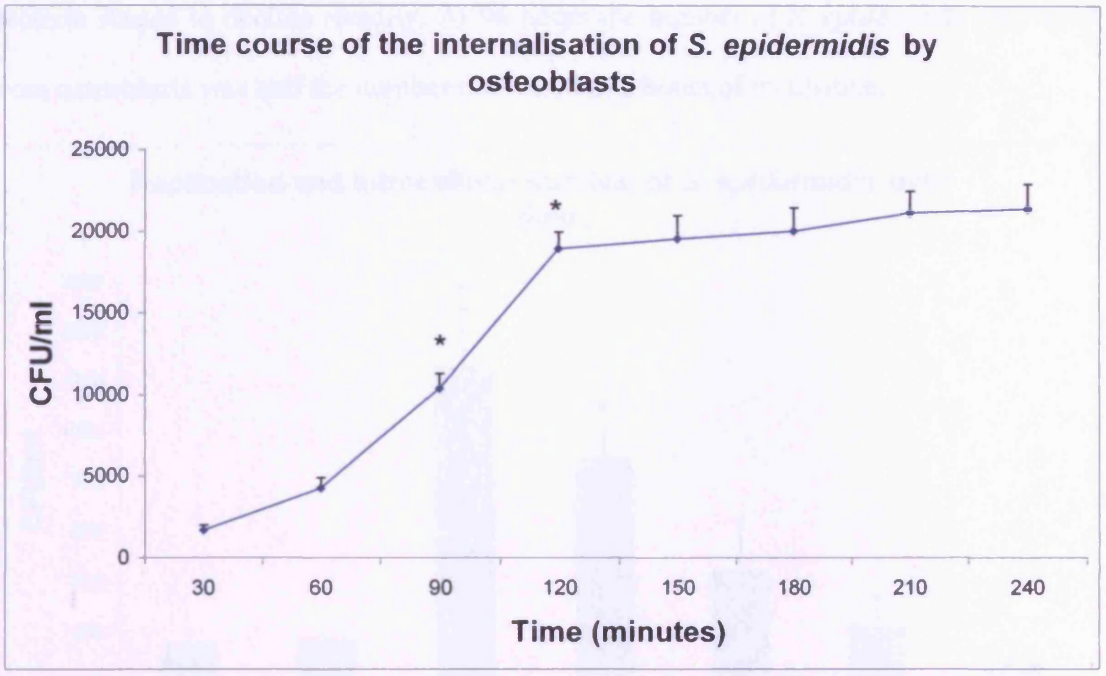

Figure 3-3: Bacterial colony-forming units per millilitre of *S. epidermidis* strain 19 internalised by osteoblasts at different time points (30 to 240 minutes) at MOI of 1:1. Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. * P value < 0.01.

3.3.4 *S. epidermidis* intracellular replication and/or persistence

The internalisation assay was performed at a MOI of 1:1 to determine if *S. epidermidis* replicated and/or survived intracellularly over long periods of time. The gentamicin protection internalisation assay was performed as described in chapter 2, section 2.5.2. Osteoblasts were washed three times with DMEM then 1 ml of DMEM containing 100µg/ml gentamicin was added. Osteoblasts were reincubated at 37°C in a humidified atmosphere containing 5% CO_2/ 95% air and then lysed at various time points (2 to 96 hours). The results of this experiment showed that the number of intracellular *S. epidermidis* recovered in the presence of gentamicin at 12 hours was 6-fold (P <0.01) higher than at 2 or 4 hours (figure 3-4). After 24 hours the number of intracellular

bacteria started to decline steadily. At 96 hours the number of *S. epidermidis* recovered from osteoblasts was half the number recovered at 2 hours of incubation.

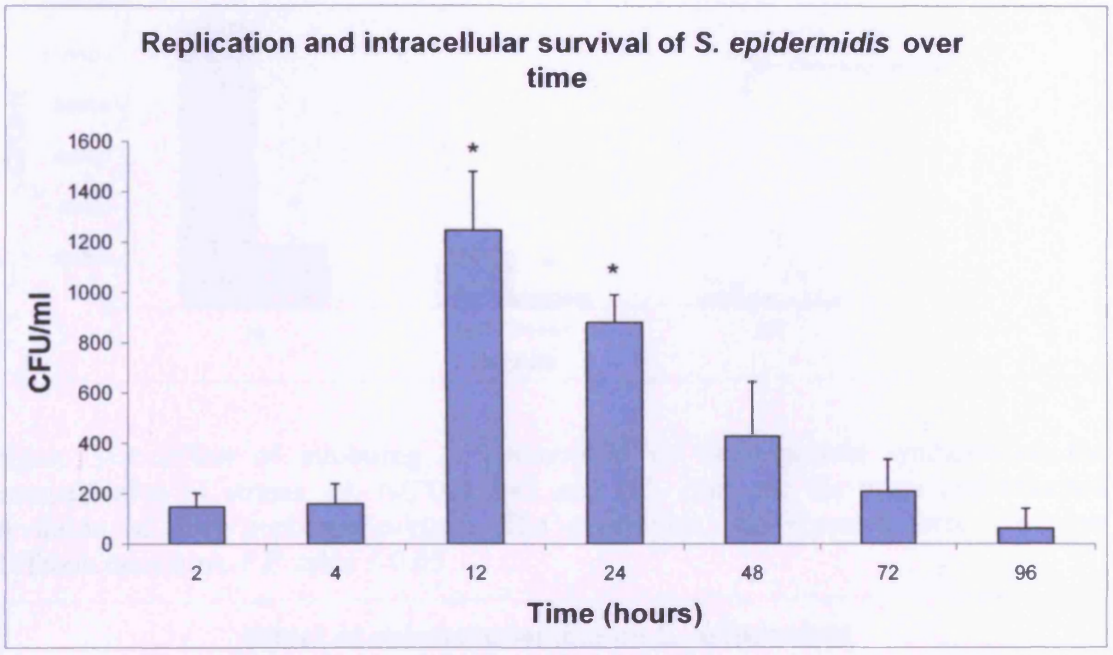

Figure 3-4: Intracellular replication and survival of *S. epidermidis* strain 19 within osteoblasts. Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. * P value < 0.01.

3.3.5 Bacterial de novo protein synthesis and internalisation

It has been reported that bacterial de novo protein synthesis is important in the internalisation of *Klebsiella pneumonia* by epithelial cells(Oelschlaeger and Tall, 1997). The importance of *S. epidermidis* de novo protein synthesis in the internalisation of bacteria by osteoblasts was investigated using chloramphenicol (inhibitor of bacterial cell protein synthesis, see section 1.6.4.1, chapter 1).

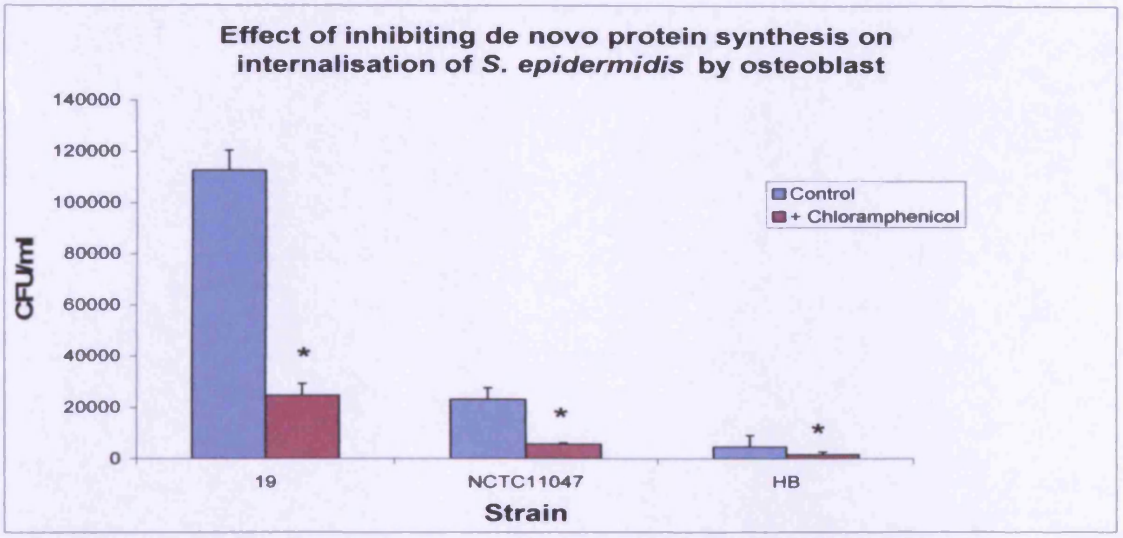

Figure 3-5: Effect of inhibiting *S. epidermidis* de novo protein synthesis on the internalisation of strains 19, NCTC11047 and HB. Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. * P value < 0.05.

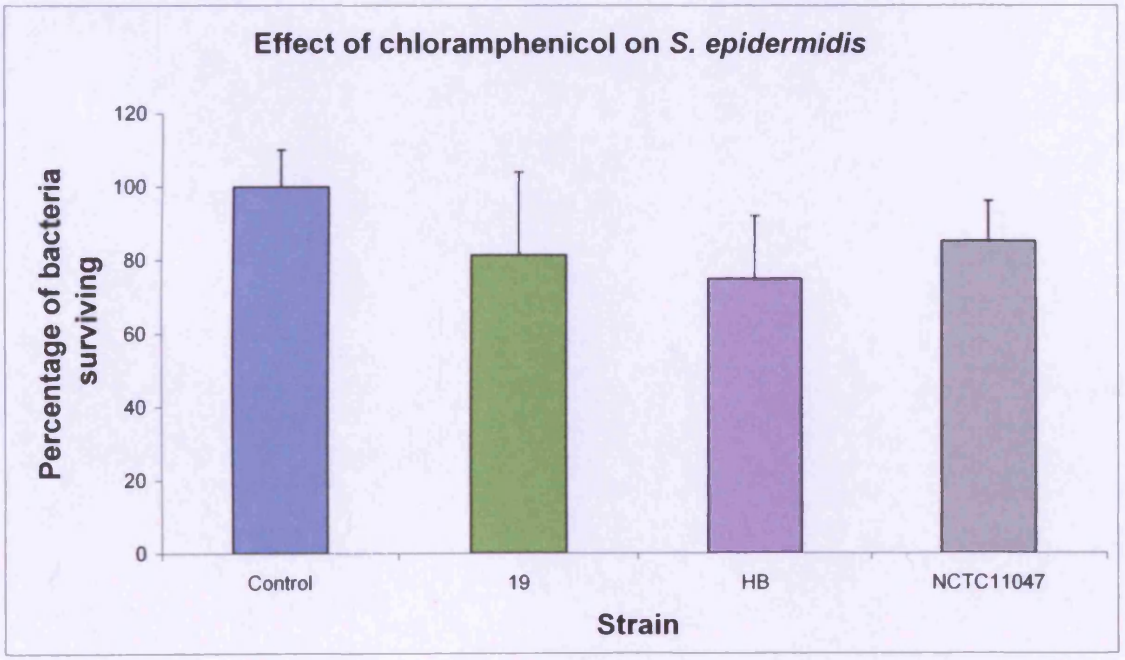

Figure 3-6: Effect of chloramphenicol on the viability of *S. epidermidis* strains 19, NCTC11047 and HB. Data are the mean and standard deviation of three replicate cultures.

S. epidermidis was resuspended in a growth medium containing chloramphenicol at a concentration of 10μg/ml then co-cultured with a confluent monolayer confluent

monolayer of osteoblasts. The internalisation of *S. epidermidis* strains 19, HB and NCTC11047 was reduced ($P < 0.01$) in the presence of chloramphenicol by 78, 67 and 72% respectively (figure 3-5). At the concentration used, chloramphenicol had no significant effect on bacterial cell viability as shown in figure 3-6.

3.3.6 The role of tyrosine kinases in the internalisation of *S. epidermidis* by osteoblasts

Genistein is a specific inhibitor of eukaryotic tyrosine protein kinases (TPK) and acts by inhibiting binding of ATP to the protein kinase (Almeida et al., 2000).

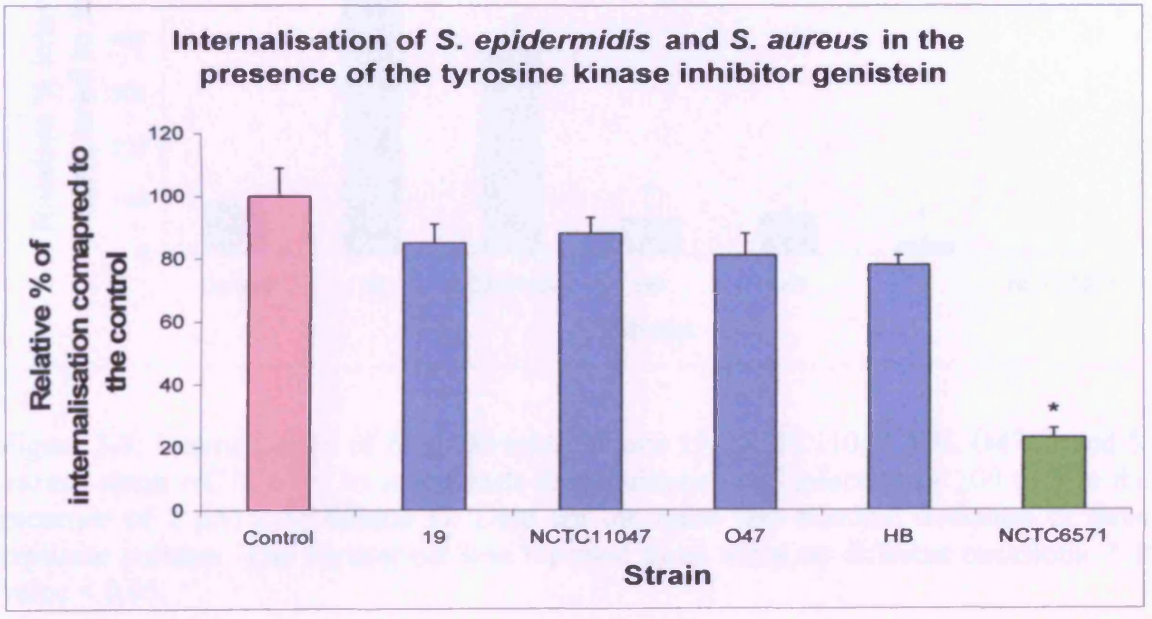

Figure 3-7: Bacterial colony-forming units per millilitre of different *S. epidermidis* strains and one *S. aureus* strain internalised by osteoblasts in the presence of the tyrosine kinase inhibitor genistein. Data are the mean and standard deviation of three replicate cultures. This graph is a representative experiment of three replicates. * P value < 0.01.

Pretreatment of osteoblasts with genistein had no significant effect on the internalisation of different *S. epidermidis* strains by osteoblasts (figure 3-7). In contrast internalisation of *S. aureus* was reduced by 80% ($P < 0.01$).

3.3.7 The effect of cytochalasin D on the internalisation of *S. aureus* and *S. epidermidis* by osteoblasts

The effect of cytochalasin D on the internalisation of five strains of *S. epidermidis* and one strain of *S. aureus* was examined.

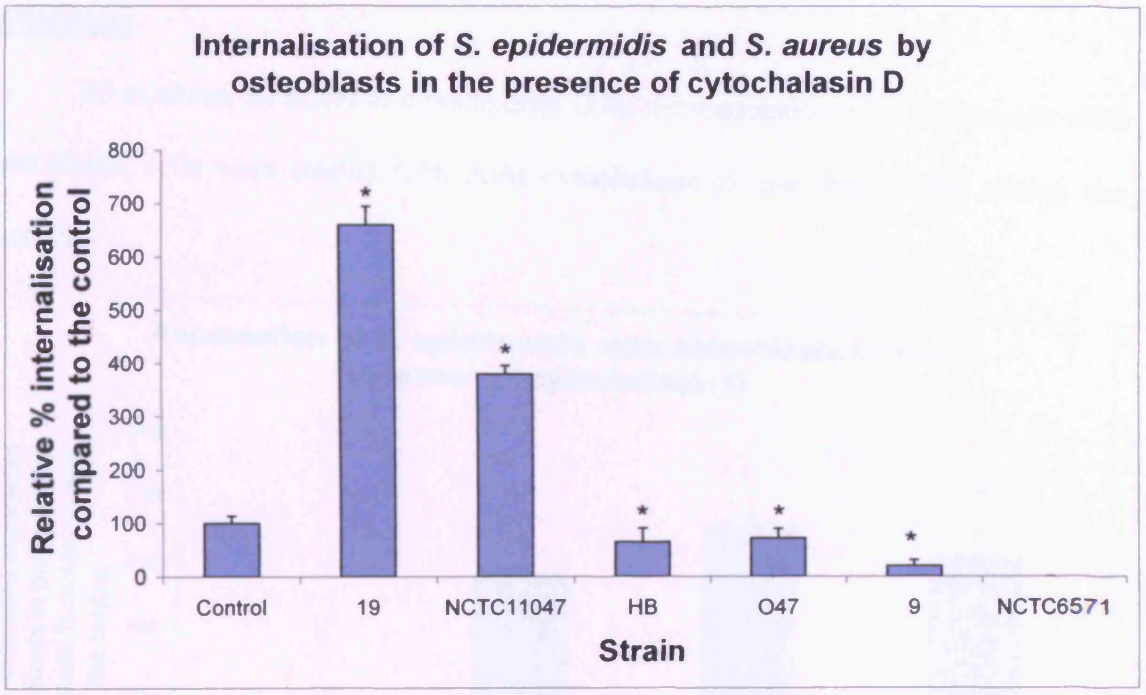

Figure 3-8: Internalisation of *S. epidermidis* strains 19, NCTC11047, HB, O47, 9 and *S. aureus* strain NCTC6571 by osteoblasts at a multiplicity of infection of 200 to 1 in the presence of 2 μM cytochalasin D. Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. * P value < 0.05.

The effect of cytochalasin D varied depending on the strain examined. Internalisation of *S. epidermidis* strains NCTC11047 and 19 was increased by 3.8 and 6.6 fold ($P < 0.01$) respectively in the presence of cytochalasin D, while internalisation of strains O-47, HB, and 9 was inhibited by 28, 25 and 80 % respectively (figure 3-8). In contrast, cytochalasin D completely blocked ($P < 0.01$) internalisation of *S. aureus* strain

NCTC6571 by osteoblasts (figure 3-8). These results suggest that *S. epidermidis* and *S. aureus* utilise different ways in their internalisation by osteoblasts.

3.3.8 The effect of cytochalasin D on the association of *S. epidermidis* with osteoblasts

To examine the effect of cytochalasin D on the association of *S. epidermidis* with osteoblasts, cells were treated with 2μM cytochalasin D one hour before adding the bacteria.

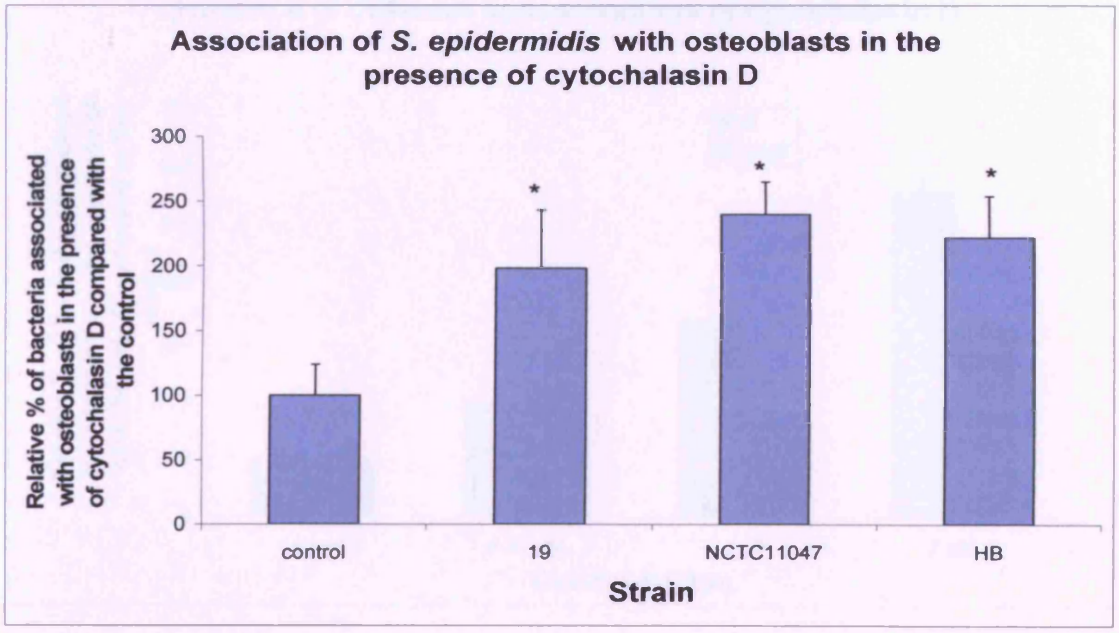

Figure 3-9: Relative percentage of *S. epidermidis* strains 19, NCTC11047 and HB associated with osteoblasts at an MOI of 200:1 in the presence of cytochalasin D. Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. * P value < 0.05.

Association of *S. epidermidis* strain 19, NCTC11047 and HB with osteoblasts increased by 2, 2.4 and 2.2-fold ($P < 0.01$) respectively relative their association with osteoblasts in the presence of no cytochalasin D (figure 3-9).

3.3.9 The effect of different cytochalasin D concentrations on internalisation of *S. epidermidis* by osteoblasts

To investigate the effect of different concentrations of cytochalasin D on internalisation of *S. epidermidis* by osteoblasts, cells were treated with three different concentrations of cytochalasin D, 0.25 μM, 0.5 μM and 2μM. The increase in the numbers of *S. epidermidis* strain 19 internalised by osteoblasts in the presence of cytochalasin D was dose-dependent (figure 3-10).

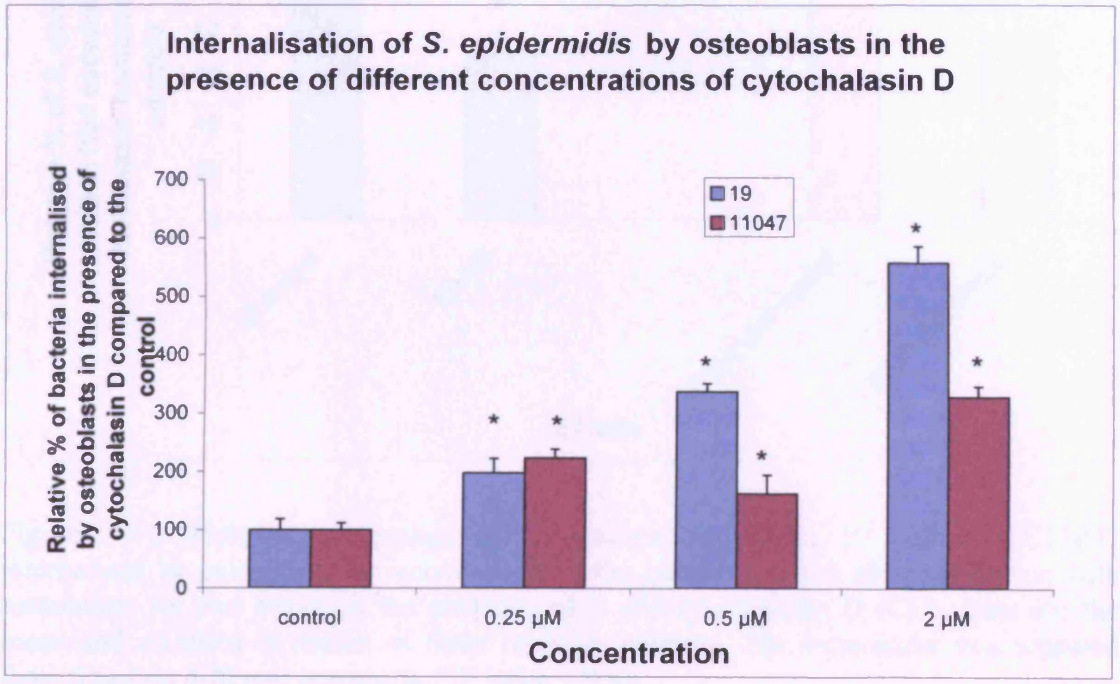

Figure 3-10: Internalisation of *S. epidermidis* strains 19 and NCTC11047 by osteoblasts at an MOI of 200:1 in the presence of different concentrations of cytochalasin D (0.25μM, 0.5μM and 2μM). Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. * P value < 0.05.

3.3.10 Effect of cytochalasin D on the escape of *S. epidermidis* from osteoblasts

After incubating *S. epidermidis* strains 19 and NCTC11047 with osteoblasts for two hours the effect of cytochalasin D on the escape of these bacteria from within the osteoblasts was examined.

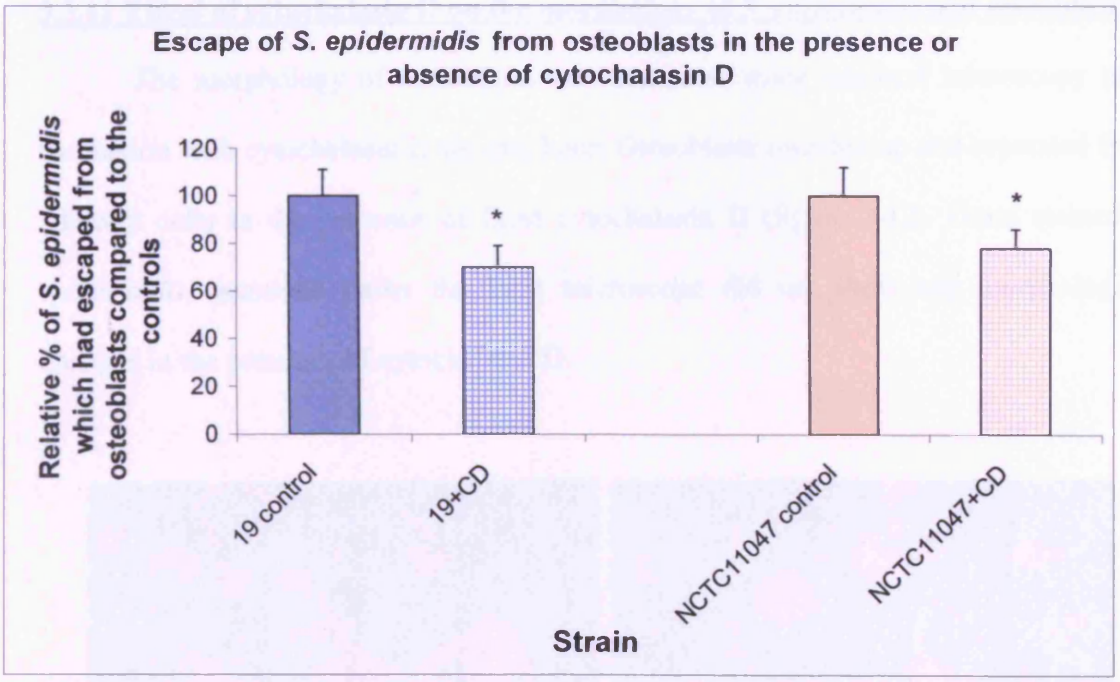

Figure 3-11: Relative percentage of *S. epidermidis* strains 19 and NCTC11047 internalised by osteoblasts or recovered from the culture medium after incubation with osteoblasts for two hours in the presence of 2 μM cytochalasin D (CD). Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. * P value < 0.05.

Bacterial cell numbers in the culture supernatant were compared to the numbers from control bacterial cultures where no cytochalasin D was added. Figure 3-11 shows that the numbers of *S. epidermidis* strains 19 and NCTC11047 which escaped from within the osteoblasts were 30% and 22% less than those that escaped from osteoblasts in the control wells where no cytochalasin D was added ($P < 0.05$). The percentage of bacteria which escaped was calculated by dividing the number of *S. epidermidis* recovered in the

culture medium by the total number of bacteria including those bacteria in the culture medium and those that had been internalised.

3.3.11 Effect of cytochalasin D on the morphology of *S. epidermidis* and osteoblasts

The morphology of osteoblasts was examined using confocal microscopy after incubation with cytochalasin D for one hour. Osteoblasts rounded up and separated from adjacent cells in the presence of 2μM cytochalasin D (figure 3-12). Gram stained *S. epidermidis* examined under the light microscope did not show any morphological changes in the presence of cytochalasin D.

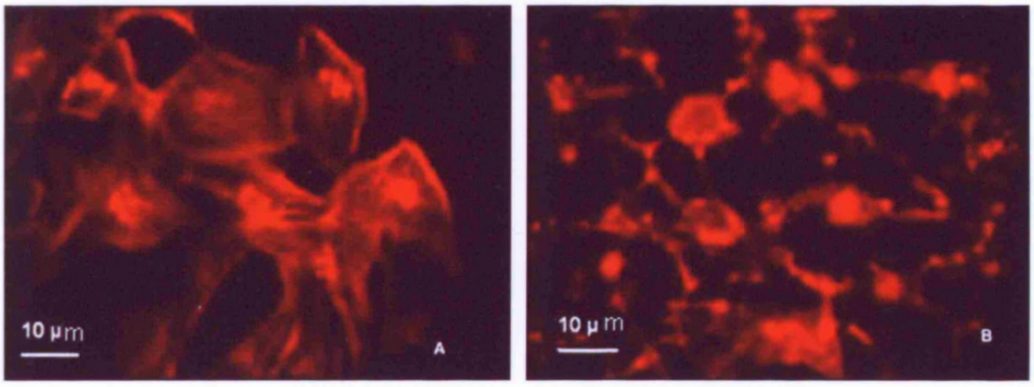

Figure 3-12: Confocal microscopic images of normal osteoblasts (A) or osteoblasts treated with cytochalasin D (B). Osteoblasts were stained with Rhodamine-conjugated phalloidin at a concentration of 1 unit / ml. Magnification is 63x.

3.3.12 The effect of jasplakinolide and latrunculin B on the internalisation of *S. epidermidis* by osteoblasts

To examine the effect of stabilising host cell microfilaments on the internalisation of *S. epidermidis* by osteoblasts, jasplakinolide was used at concentrations based on previous studies (Mizuno et al., 2002) and added to osteoblasts one hour before adding

the bacteria. There was a significant (P <0.05) increase (14-fold) in the numbers of *S. epidermidis* recovered from inside osteoblasts in the presence of jasplakinolide.

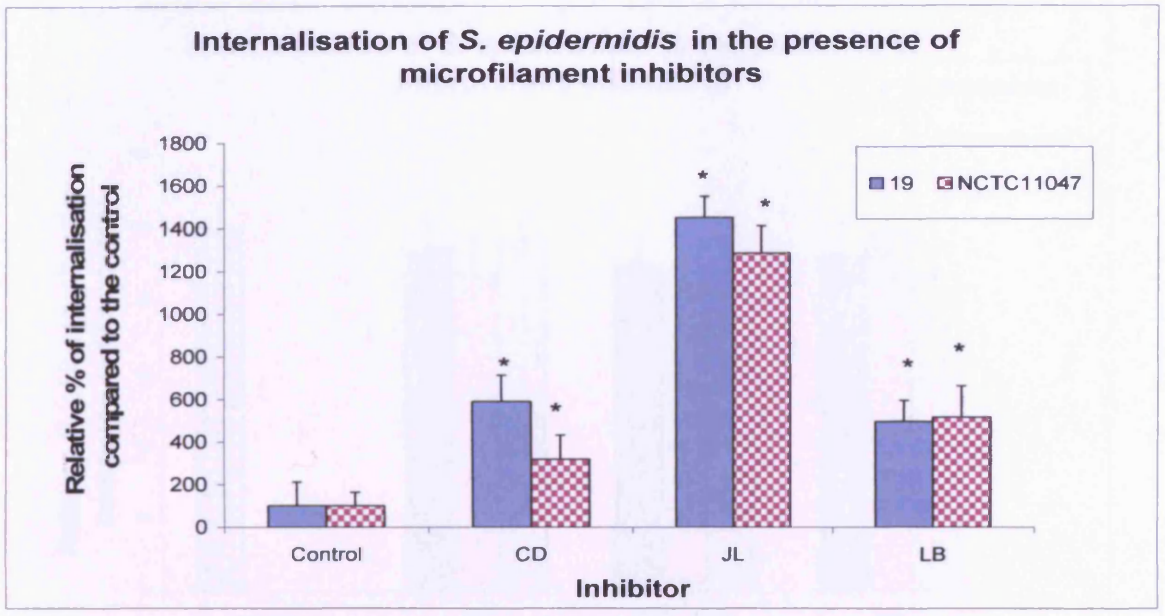

Figure 3-13: The relative percentage of internalisation of *S. epidermidis* strains 19 and NCTC11047 by osteoblasts in the presence of cytochalasin D (CD) or latrunculin B (LB) or jasplakinolide (JL). Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. The results were compared to the internalisation of *S. epidermidis* by osteoblasts in the presence of no inhibitors. * P value < 0.05.

Depolymerisation of host cell microfilaments using latrunculin B which permanently disrupts microfilaments organization and cytochalasin D which inhibits microfilament depolymerisation increased the numbers of *S. epidermidis* strains 19 and NCTC11047 recovered from inside osteoblast by 4.9 (P< 0.05) and 5.8 (P< 0.05) fold (figure 3-13).

3.3.13 Effect of microtubule depolymerisation or stabilisation on the internalisation of *S. epidermidis* by osteoblasts

In addition to microfilaments, microtubules are major components of the cytoskeleton. They can be depolymerised by colchicine or nocodazole and stabilised by

taxol. To examine the role of microtubules in the internalisation of *S. epidermidis* by osteoblasts, internalisation assays were carried out in the presence of these inhibitors.

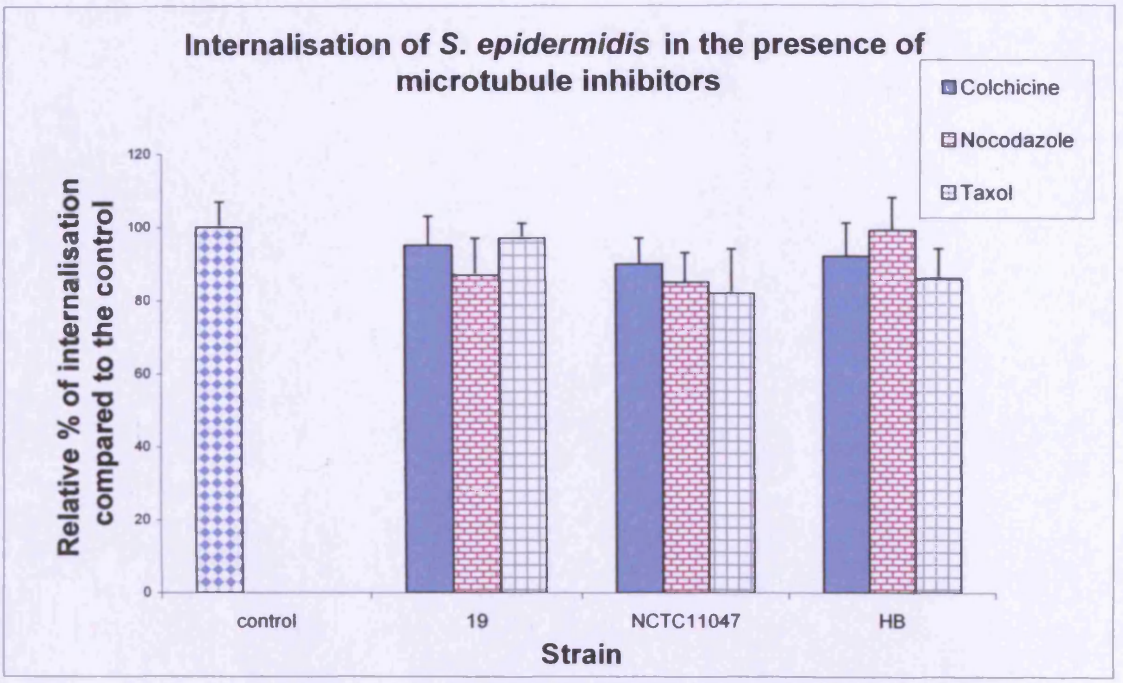

Figure 3-14: Internalisation of *S. epidermidis* strains 19, NCTC11047 and HB by osteoblasts at a multiplicity of infection of 200 to 1 in the presence of either colchicine or nocodazole or taxol. Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. The results were compared to the internalisation of these strains in the absence of inhibitors.

Microtubule depolymerisation or stabilisation had no effect on the internalisation of *S. epidermidis* by osteoblasts (figure 3-14). These results suggest that microtubules did not play a role in the mechanism of internalisation of *S. epidermidis* by osteoblasts.

3.3.14 The role of endosome acidification and receptor mediated endocytosis in the internalisation of *S. epidermidis* by osteoblasts

To block endosome acidification, internalisation assays were conducted in the presence of monensin. The uptake of *S. epidermidis* strain 19, HB and NCTC11047 by osteoblasts was inhibited by 35, 75 and 63% respectively (figure 3-15).

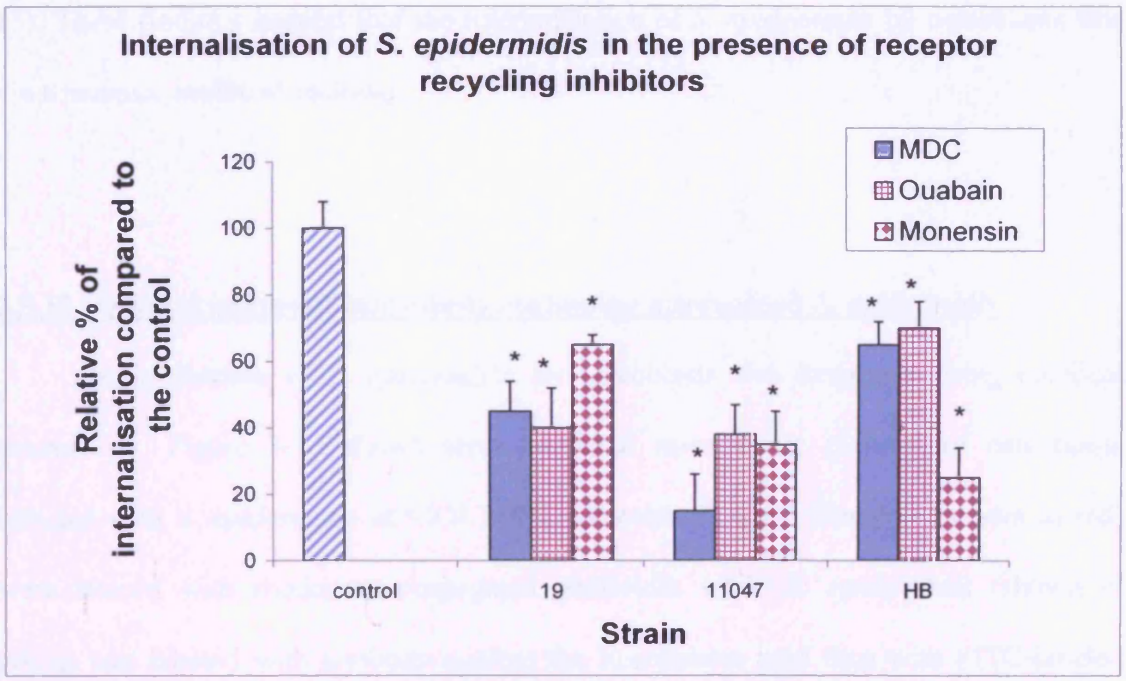

Figure 3-15: Internalisation of *S. epidermidis* strains 19, NCTC11047 and HB by osteoblasts at an MOI of 200:1 in the presence of monensin or ouabain or monodansylcadaverine (MDC). Data are the mean and standard deviation of three replicate cultures. The experiment was repeated three times on different occasions. * P value < 0.05.

The formation of coated pits can be inhibited by monodansylcadaverine and ouabain (Dorn et al., 1998; Jevon et al., 1999; Biswas et al., 2000). Preincubation of osteoblasts with monodansylcadaverine which inhibits transglutaminase and interferes with receptor recycling reduced internalisation of *S. epidermidis* strains by osteoblasts. The greatest effect was seen with strain NCTC11047 (84% reduction) (figure 3-15). The addition of ouabain which blocks Na+/K+ ATPase causing arrest of coated pit formation by inhibiting the interaction of clathrin and adapter protein interaction reduced internalisation of *S. epidermidis* by osteoblasts. The degree of inhibition of internalisation was different for each of the strains and varied from 35 to 61 % (figure 3-

15). These findings suggest that the internalisation of *S. epidermidis* by osteoblasts was via a receptor mediated pathway.

3.3.15 Confocal images of osteoblasts containing internalised *S. epidermidis*

Internalisation of *S. epidermidis* by osteoblasts was examined using confocal microscopy. Figure 3-16 shows serial confocal microscopic pictures of osteoblasts infected with *S. epidermidis* at MOI 200:1. Osteoblast's actin filaments (shown in red) were stained with rhodamine-conjugated phalloidin while *S. epidermidis* (shown in green) was labeled with antibody against the lipoteichoic acid then with FITC-labeled secondary antibody. Details of the labelling and staining procedures can be seen in section 2.2.2 and section 2.3.3.2, chapter 2. The direction of confocal scanning was from the top of the osteoblast to the bottom. Houalet-Jeanne et al have used the serial sections method to show internalised *Porphyromonas gingivalis* by epithelial cells (Houalet-Jeanne et al., 2001). In figure 3-16 the white arrows follow a single osteoblast through the whole sections. When reaching the middle sections such as section 9 and 10 some intracellular *S. epidermidis* (yellow arrows) can be seen. A better way of showing intracellular *S. epidermidis* will be the construction of a three dimensional picture from a series of sections through osteoblasts containing *S. epidermidis*. It was difficult to construct a 3D picture with the available software at our laboratory.

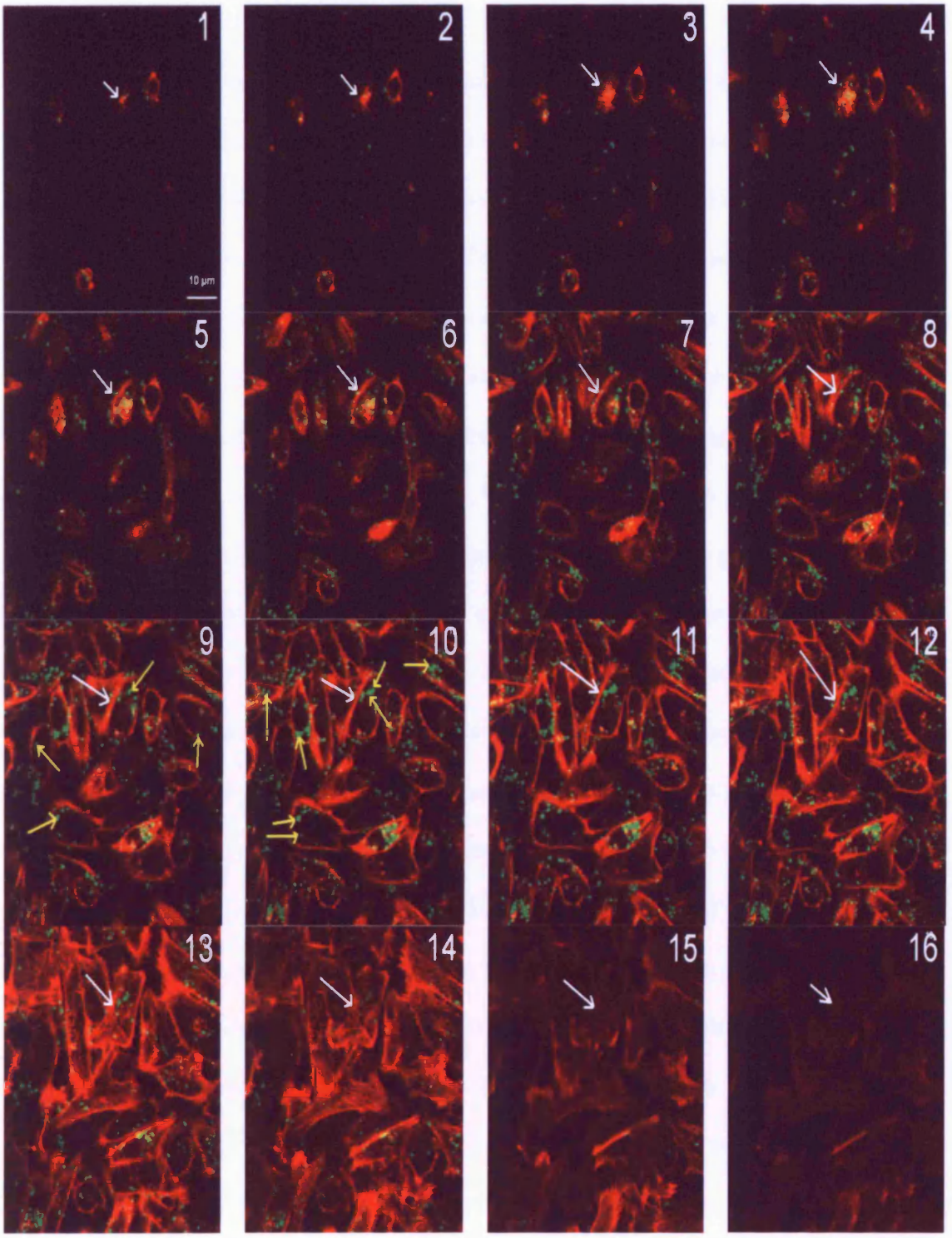

Figure 3-16: Confocal microscopic images of *S. epidermidis* internalised by osteoblasts. Osteoblast actin filaments were stained with rhodamine-conjugated phalloidin (shown in red) and *S. epidermidis* (shown in green) was immunolabelled with monoclonal anti-lipoteichoic acid and FITC labelled anti-mouse antibody. The sections are going through the osteoblasts from the top to the bottom. Section thickness 0.5 µm. the white bar measures 10µm.

3.4 Discussion

In this study we examined the capacity of *S. epidermidis* to be internalised by bone cells, osteoblasts. The results of the investigation have shown that osteoblasts are able to internalise some *S. epidermidis* strains such as 19, NCTC11047, HB, 9, and O47 but not others such as strain NCTC11964. The magnitude of internalisation of strain 19 was similar to that reported for the internalisation of some *S. aureus* strains (Jevon et al., 1999). However different *S. epidermidis* strains were internalised by osteoblasts to different extents and *S. epidermidis* strain 19 was internalised in greater numbers than strains NCTC11047, HB, O47, or strain 9. The difference in the capacity of *S. epidermidis* strains to be internalised by osteoblasts may affect the prognosis of disease. Although adherence of microbial pathogens to host cells is considered a primary step in the pathogenesis of many infections (Biswas et al., 2000), in our study the capacity to be internalised did not correlate with the capacity to bind to osteoblasts. For example, *S. epidermidis* strains NCTC11047 and HB associated in higher numbers with osteoblasts than strain 19 but were internalised to a lesser extent. *S. epidermidis* remained viable for 96 hours after internalisation by osteoblasts. However the number of intracellular bacteria decreased over time (figure 3-3). There are a number of possible explanations for the decrease in the bacterial numbers. It could have been due to the death of the osteoblasts or leakage of gentamicin into the bone cells killing the intracellular bacteria or death of the intracellular bacteria or escape of the bacteria from the bone cells. The multiplicity of infection also had an effect on the number of intracellular bacteria. Lowering the MOI decreased the number of intracellular bacteria per osteoblast, whereas raising the MOI increased the number of intracellular bacteria per cell (figure 3-2). The maximum internalisation efficiency for *S. epidermidis* in the present study was 1.3% of the initial

inoculum, a percentage significantly lower than those indicated for some human invasive bacterial pathogens, such as *Salmonella typhimurium* (Huang et al., 1998b), which showed 48.9%. However the level found for *S. epidermidis* is higher than that reported for some species of *Campylobacter jejuni* (0.1-0.2%) (Hu and Kopecko, 1999). At a higher MOI such as 1000:1 the numbers of intracellular bacteria started to decline and the internalisation efficiency was decreased to 0.3%. These results suggest that internalisation of *S. epidermidis* by osteoblasts is a process mediated by receptor-ligand interaction, since this type of interactions is characterised by saturability (Jiang et al., 2003). The relation between MOI and internalisation should be borne in mind in future studies on internalisation of *S. epidermidis* by host cells since the MOI chosen represents a compromise between number of internalised bacteria and internalisation efficiency. Other factors such as de novo protein synthesis by bacteria have been reported to affect internalisation of some bacteria by host cells. For example bacterial de novo protein synthesis has been reported to be essential in *Klebsiella pneumonia* internalisation by epithelial cells (Oelschlaeger and Tall, 1997) but not for uptake of *Yersinia enterocolitica* by epithelial cells (Oelschlaeger and Tall, 1996). The use of chloramphenicol, which inhibits bacterial de novo protein synthesis, in the internalisation assays of *S. epidermidis* by osteoblasts showed that de novo protein synthesis played a role in the internalisation process of this bacterium by osteoblasts. In the presence of chloramphenicol internalisation of *S. epidermidis* strains 19, NCTC11047 and HB was reduced by 78, 72 and 67% respectively (figure 3-5).

Many bacterial pathogens activate host cell signal transduction pathways during their uptake. In eukaryotic cells, extracellular signals are transduced into the cell through phosphorylation of key proteins by protein kinases such as protein kinase C (PKC) and

tyrosine protein kinases (TPK). A strong link between bacterial internalisation and host cell signalling through protein kinases (PTK) has been well documented in several host cell-bacterium interactions (Dziewanowska et al., 1999; Almeida et al., 2000; Fowler et al., 2003). Since some bacterial pathogens elicit their own internalisation through induction of host cell cytoskeleton rearrangement (Lamaze et al., 1997; Dramsi and Cossart, 1998), we investigated if TPK activity was necessary for internalisation of *S. epidermidis*. *S. aureus* was used as a positive control, since internalisation of this bacterium by osteoblasts has been reported to be inhibited by tyrosine protein kinase inhibitors (Alexander and Hudson, 2001). Unlike the case with *S. aureus*, tyrosine protein kinases did not seem to be important in the internalisation of *S. epidermidis* by osteoblasts (figure 3-7).

Cytochalasin D is fungal metabolite which has a low molecular weight. It binds to actin and causes depolymerisation of host cell microfilament. It has been shown that internalisation of *S. aureus* by osteoblasts can be blocked by the use of cytochalasin D (Jevon et al., 1999). The data presented in this chapter show that the effect of cytochalasin D on the internalisation of *S. epidermidis* by osteoblasts varied depending on the strain examined. Internalisation of *S. epidermidis* strains 9, O-47 and HB by osteoblasts was inhibited by cytochalasin D. This inhibitory effect was not as great as that seen with *S. aureus* where cytochalasin D blocked internalisation by 99 to 100% (figure 3-8). In contrast the internalisation of *S. epidermidis* strains 19 and NCTC11047 by osteoblasts was increased by the addition of cytochalasin D. Internalisation of *Salmonella typhimurium* by enterocytes has also been shown to be increased in the presence of cytochalasin D (Wells et al., 1998). *Actinobacillus actinomycetemcomitans* also exhibited

an enhanced ability to enter epithelial cells in the presence of cytochalasin D (Brissette and Fives-Taylor, 1999). Examination of osteoblasts treated with cytochalasin D showed that the cells were distorted; rounding and separating from adjacent cells in the confluent monolayer (figure 3-12). These morphological changes may affect the surface area of the cell exposed to *S. epidermidis*. Cytochalasin D had no noticeable effect on the viability of *S .epidermidis* or osteoblasts. Wells et al, reported that rounding of enterocytes by cytochalasin D exposes the lateral surface of the cell which is an important area for internalisation of *Salmonella typhimurium* and *Proteus. mirabilis* (Wells et al., 1998). The effect of cytochalasin D on the internalisation of *S. epidermidis* by osteoblasts was dose dependent. One possible explanation for the increase in the internalisation of *S. epidermidis* strains 19 and NCTC11047 by osteoblasts could be related to an increase in adherence of *S. epidermidis* to osteoblasts in the presence of cytochalasin D. Although cytochalasin significantly increased the association of *S. epidermidis* strains 19 and NCTC11047 with osteoblasts it is also increased the adherence of strain HB. Taken together these data suggest that an increase in the adherence of *S. epidermidis* strains to cytochalasin D treated osteoblasts may not be responsible for the increased numbers of bacteria internalised. To examine the effect of cytochalasin D on *S. epidermidis* externalisation or escape from osteoblasts, these cells were treated with cytochalasin D after a standard gentamicin protection assay. Treating the osteoblasts with cytochalasin D reduced the number of bacteria recovered in the supernatant when compared to the numbers of bacteria recovered from the supernatant in the control wells where no cytochalasin D was added (figure 3-11). This suggests that cytochalasin D prevents bacteria from escaping from osteoblasts after they have been internalised and that this could be a reason that greater numbers of bacteria are found intracellularly. We used

other compounds that inhibited microfilaments to rule out the possibility of any non-specific action of cytochalasin D on the process of internalisation of *S. epidermidis* by osteoblasts. Jasplakinolide which is a potent stabiliser of microfilaments increased the numbers of *S. epidermidis* recovered intracellularly from osteoblasts by 12-fold compared to the number of intracellular bacteria where no jasplakinolide was added (figure 3-13). Depolymerising microfilaments using latrunculin B also caused an increase in the numbers of *S. epidermidis* strain 19 found inside osteoblasts (figure 3-13). These findings show that stabilisation or depolymerisation of microfilaments increases the number of *S. epidermidis* strain 19 found inside osteoblasts.

In addition to microfilaments, microtubules are also major components of the cytoskeleton. It has been shown that internalisation of *S. aureus* can be inhibited by 40 and 55% by colchicine and nocodazole respectively (Jevon et al., 1999). These inhibitors disrupt the microtubule networks of the host cell and cause their depolymerisation. It has been reported that when internalisation depends on microtubules and microfilaments, the inhibitory effect of depolymerising microfilaments using inhibitors is more profound than depolymerising microtubules (Oelschlaeger and Tall, 1997). In the experiments presented here, internalisation of *S. epidermidis* by osteoblasts was not affected by depolymerising or stabilising microtubules (figure 3-14). This indicates that microtubules are not critical in internalisation of *S. epidermidis* by osteoblasts. The difference in the effect of microfilament and microtubule inhibitors on the internalisation of *S. epidermidis* and *S. aureus* by osteoblasts suggest that these two organisms gain access into osteoblasts via different pathways.

The use of monodansylcadaverine, an inhibitor of clathrin-dependent receptor mediated endocytosis, decreased the numbers of intracellular *S. epidermidis* strain NCTC11047 by nearly 85% (figure 3-15). Internalisation of *S. epidermidis* strains 19 and HB by osteoblasts was also inhibited, by 54% and 35% respectively, by monodansylcadaverine. Ouabain which is another inhibitor of coated pit formation reduced internalisation of *S. epidermidis* by osteoblasts by 35 to 61%. Monensin which affects endosome acidification and receptor recycling decreased the internalisation of *S. epidermidis* strains NCTC11047, HB and 19 by 63, 75 and 35% respectively. This suggests that receptor recycling plays a role in the internalisation of *S. epidermidis* by osteoblasts.

3.5 Conclusions

Data presented in this chapter show that *S. epidermidis* can be internalised by osteoblasts and that internalisation is probably via a receptor mediated pathway. The internalisation of *S. epidermidis* by osteoblasts seems to be via a different pathway than that utilised by *S. aureus*. The finding that *S. epidermidis* can be internalised by osteoblasts may contribute to our understanding of the pathogenicity of this organism in bone disease. This may also help in the design of new drugs against infections caused by this organism. Although we have tested the role of some osteoblast structures in the uptake process, many other factors such as ligand receptor signalling and cytoskeleton proteins could be involved in the process and may require further investigation.

Chapter 4

Chapter 4

Virulence determinants involved in the internalisation of *S. epidermidis* by osteoblasts

4.1 Introduction

Virulence factors are microbial attributes that determine an organism's capacity to cause disease (Casadevall and Pirofski, 2001). Only a few virulence factors have been identified for *S. epidermidis*. These include its ability to form biofilms and produce several extracellular products, not all of which have been well characterised (Vuong and Otto, 2002). Direct primary attachment and biofilm formation by *S. epidermidis* could be linked to surface properties such as hydrophobicity, surface charge, teichoic acid and surface proteins e.g. the autolysin AtlE. Bacterial autolysins are potentially lethal enzymes capable of hydrolysing the cell wall peptidoglycan of the bacterial cell (Foster, 1995). AtlE is a multifunctional, surface-associated protein having both enzymic and adhesive functions (Heilmann et al., 2003). A recent study has demonstrated the importance of AtlE in *S. epidermidis* pathogenicity. A *S. epidermidis* mutant strain deficient in production of autolysin has been shown to be less virulent than the wild-type parental strain in an intravascular catheter-associated infection model in rats (Rupp et al., 2001). An autolysin mutant of *S. epidermidis* also has impaired attachment to polystyrene (Heilmann et al., 1996). MSCRAMMs of *S. epidermidis* such as the fibrinogen-binding protein (Fbe) (Nilsson et al., 1998) might be important in the binding of this organism to surfaces. The gene encoding Fbe has been cloned and sequenced (Nilsson et al., 1998). It has also been named SdrG (Davis et al., 2001) and is structurally related to *S. aureus* clumping factor A (ClfA) and ClfB. It can enhance binding of *S. epidermidis* to fibrinogen (Hartford et al., 2001). Internalisation of *Streptococcus agalactiae* by

epithelial cells has been reported to be promoted by its fibrinogen binding protein (Gutekunst et al., 2004). Antibodies against *S. epidermidis* SdrG (Fbe) enhance the phagocytosis of this bacterium by macrophages (Rennermalm et al., 2004). Polysaccharide intercellular adhesin (PIA) has been proposed to be the key molecule in cell to cell adhesion. The importance of PIA as a virulence factor has been shown in a mouse model. Isogenic mutants disrubted in PIA were found to be less virulent than the wild-type parental strain (Rupp et al., 1999; Rupp et al., 2001). PIA has been show to have an important role in *S. epidermidis* biofilm formation (von Eiff et al., 2002) but its possible role in internalisation by mammalian cells has not been investigated. *S. epidermidis* produces lipase enzymes that hydrolyse lipids with long fatty acids chains (Rosenstein and Gotz, 2000). *S. epidermidis* with mutation in the accessory gene regulator (*agr*) has been shown to have a reduced lipolytic activity suggesting a role for the *agr* in the regulation of expression of the genes coding for lipase proteins (Vuong et al., 2000a). Lipases have been implicated as possible virulence factors in some bacterial infections (Rosenstein and Gotz, 2000). In *S. epidermidis* two lipases have been cloned and sequenced, the lipase GehC has been proposed to be involved in skin colonisation. The GehD lipase of *S. epidermidis* has been shown to be a bifunctional molecule, acting both as a lipase and a collagen adhesin (Bowden et al., 2002).

The data presented in chapter three showed that *S. epidermidis* can be internalised by osteoblasts via a receptor mediated pathway. In the present study, we examined a possible role for different *S. epidermidis* virulence factors in the internalisation of this organism by bone cells.

4.2 Materials and Methods

4.2.1 Internalisation assay

The gentamicin protection assay was performed as described in chapter 2, section 2.5.2.

4.2.2 Association assay

The association of *S. epidermidis* with osteoblasts was assayed as described in chapter 2, section 2.5.1.

4.2.3 Expression of recombinant FnBPB[D1-D4]

Recombinant FnBPB[D1-D4] was expressed and purified as described in chapter 2, section 2.7. The protein was run on SDS polyacrylamide gel, as described in chapter 2, section 2.8.3.

4.3 Statistics

All data are shown as the mean ± the standard deviation. Data were compared using Student's t test or Mann Whitney U test.

4.4 Results

4.4.1 Effect of a recombinant fragment of the fibronectin binding protein from *S. aureus* on the association of *S. epidermidis* with osteoblasts

FnBPB[D1-D4] was extracted and purified as described in section 4.2.3. Figure 4-1 shows the gel electrophoresis for the rFBPB[D1-D4]. The first lane is the molecular weight marker with the two reference molecular weights 27 kDa and 66 kDa. The second lane show the purified FnBPB[D1-D4] of *S. aureus*. Two bands can be seen in the second lane. A 32 kDa protein which is the FnBPB[D1-D4] and a second protein with a higher molecular weight (54 kDa). A possible explanation of the second band is being a protein dimer. Indeed another group at the Eastman working with the same protein has shown with western blot that this protein is FnBPB.

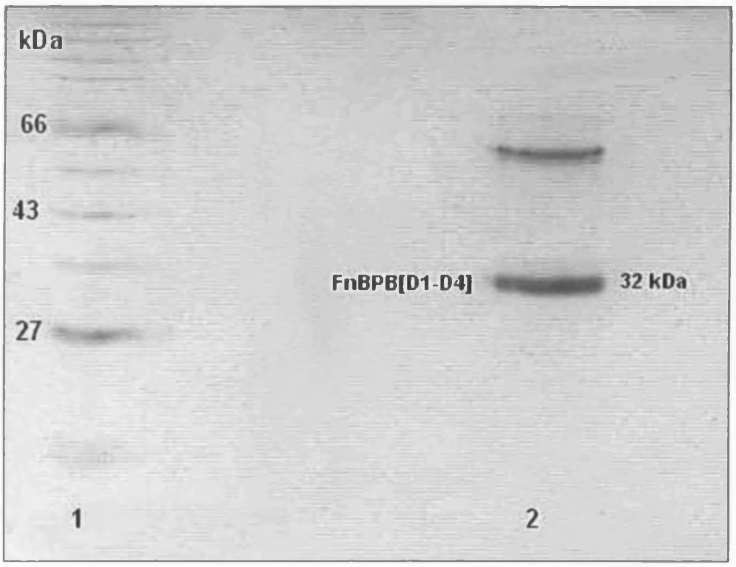

Figure 4-1: Photograph of the SDS-PAGE gel of purified rFnBPB[D1-D4]. Lane 1: marker; lane 2: rFnBPB[D1-D4].

It has been shown that the fibronectin binding proteins (FnBPs) of *S. aureus* are needed for the adhesion of this organism to bovine mammary cells (Lammers et al., 1999). FnBPs have been shown to be required for the process of internalisation of *S. aureus* by eukaryotic cells. It was proposed that the affinity of FnBP for fibronectin bound to β1 integrins would result in activation of host cell signal transduction pathways, which in turn would lead to actin-mediated phagocytosis of adherent bacteria (Haggar et al., 2003). To examine the effect of a recombinant fragment of the *S. aureus* fibronectin binding protein on association of *S. epidermidis* with osteoblasts we incubated osteoblasts with rFnBPB[D1-D4] for one hour before adding the bacteria. The results show that the association of *S. epidermidis* strains 19 and NCTC11047 was not affected by the presence of rFnBPB[D1-D4] (figure 4-2).

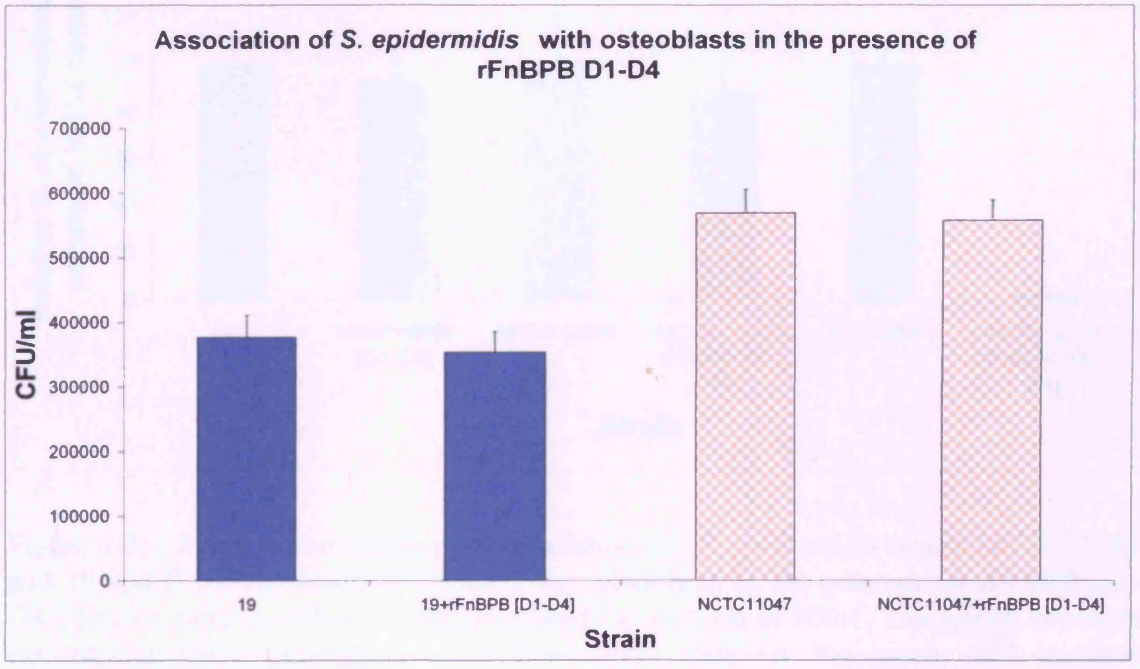

Figure 4-2 : Association of *S. epidermidis* strains NCTC11047 and 19 with osteoblasts in the presence of rFnBPB[D1-D4]. Strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. (CFU/ml: colony forming units per milliliter).

4.4.2 Effect of a recombinant fragment of the fibronectin binding protein from *S. aureus* on the internalisation of *S. epidermidis* by osteoblasts

In vitro experiments have demonstrated that the fibronectin binding proteins of *S. aureus* are essential for internalisation by osteoblasts (Ahmed et al., 2001). To determine if a similar fibronectin binding protein in *S. epidermidis* is involved in the internalisation of this bacterium by osteoblasts, internalisation assays were carried out in the presence of recombinant fibronectin binding protein rFnBPB[D1-D4] (figure 4-3).

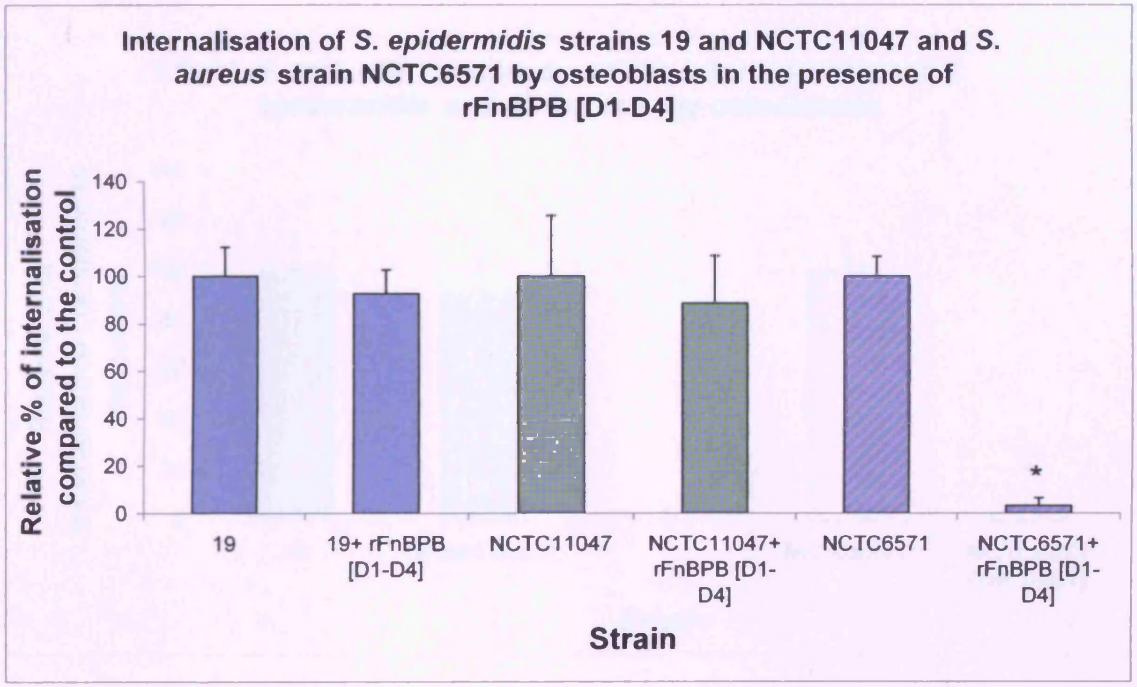

Figure 4-3: Relative percentage of internalisation of *S. epidermidis* strains NCTC11047 and 19 and *S. aureus* strain NCTC6571 by osteoblasts in the presence of rFnBPB[D1-D4]. Strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures.

The presence of rFnBPB[D1-D4] did not inhibit the internalisation of *S. epidermidis* strains 19 or NCTC11047 by osteoblasts suggesting that *S. epidermidis* did not express a

protein similar to the *S. aureus* FnBPs that was involved in internalisation of this bacterium by osteoblasts.

4.4.3 α5β1 integrin is not involved in the internalisation of *S. epidermidis* by osteoblasts

Internalisation of *S. aureus* by mammalian cells has been described to occur via a bridging model in which the organism binds to fibronectin, which in turn binds to the α5β1 integrin on the host cell surface (Peacock et al., 1999; Lammers et al., 1999; Alexander and Hudson, 2001; Ahmed et al., 2001; Jett and Gilmore, 2002).

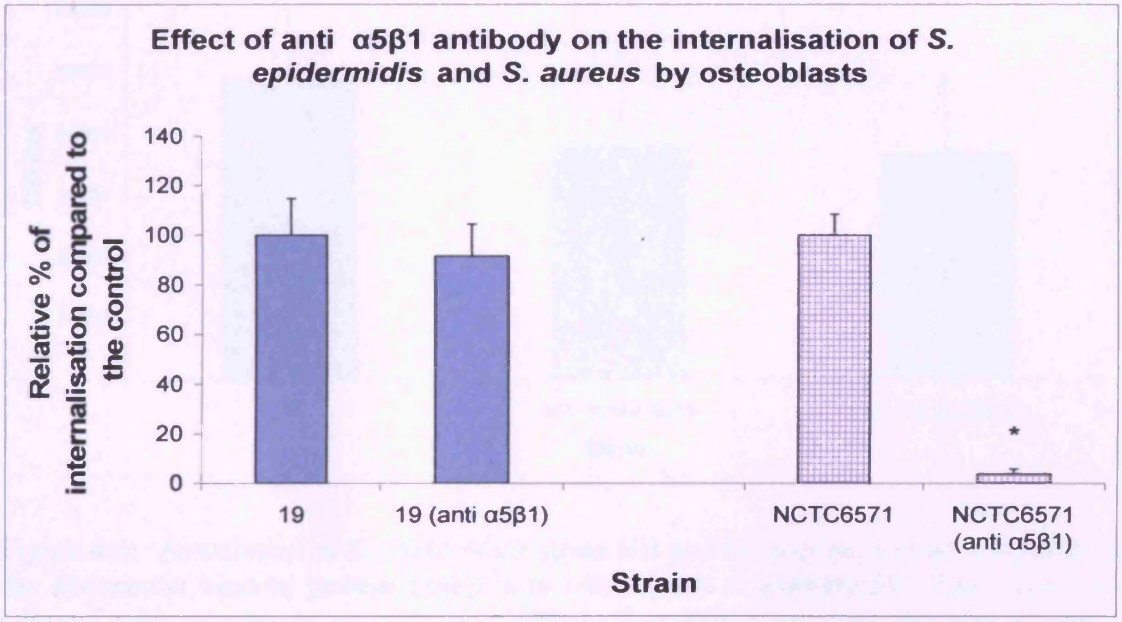

Figure 4-4: The effect of anti-α5β1antibody on the internalisation of *S. epidermidis* strain 19 by osteoblasts. *S. epidermidis* was co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. * P value < 0.05.

We examined the involvement of the α5β1 integrin in the internalisation of *S. epidermidis* by osteoblasts. The results in figure 4-4 demonstrate that α5β1 integrin does not participate in the internalisation of *S. epidermidis* by osteoblasts.

4.4.4 Association of *S. epidermidis* strain HB and its isogenic mutants deficient in the fibronectin binding protein Embp with osteoblasts

An isogenic mutant of *S. epidermidis* strain HB with a disruption at the start of the gene for Embp (HB-Embp3349) and an isogenic mutant with a disruption in the 3' prime end of the gene coding for Embp (HB-Embp284335)(Williams et al., 2002) were examined for their capacity to bind to osteoblasts.

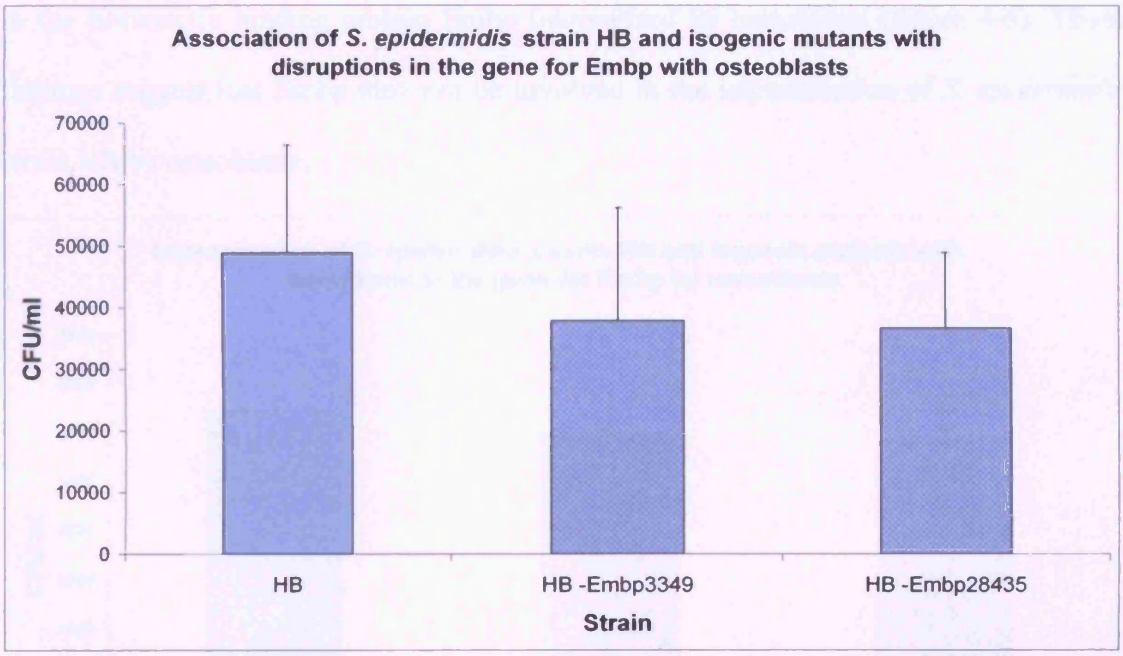

Figure 4-5: Association of *S. epidermidis* strain HB and its isogenic mutants deficient in the fibronectin binding protein Embp with osteoblasts. *S. epidermidis* strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. (CFU/ml: colony forming units per millilitre).

Figure 4-5 shows that there was no significant difference in the capacity of the isogenic mutants to associate with osteoblasts compared to the parental strain.

4.4.5 Internalisation of *S. epidermidis* strain HB and its isogenic mutants deficient in the fibronectin binding protein Embp by osteoblasts

To examine the role of *S. epidermidis* embp in the internalisation of this bacterium by epithelial cell, *S. epidermidis* strain HB and its isogenic mutants disrupted in the gene coding for Embp were used in the assays. There were no significant differences in the numbers of *S. epidermidis* strain HB and its isogenic mutants deficient in the fibronectin binding protein Embp internalised by osteoblasts (figure 4-6). These findings suggest that Embp may not be involved in the internalisation of *S. epidermidis* strain HB by osteoblasts.

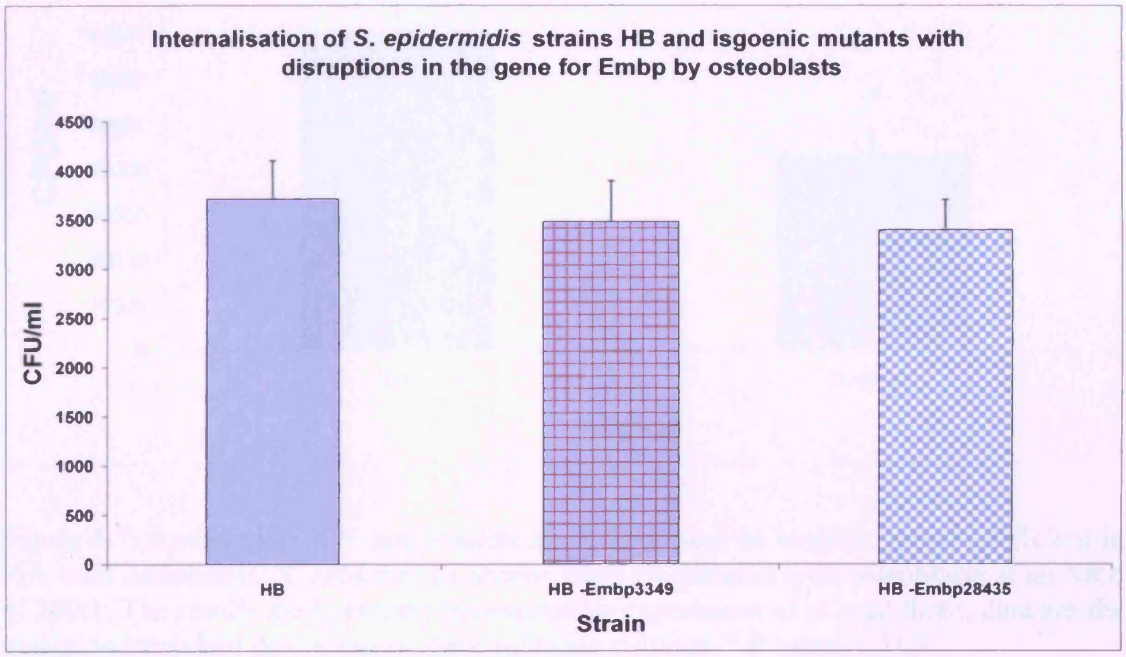

Figure 4-6: Internalisation of *S. epidermidis* strain HB and its isogenic mutants deficient in the fibronectin binding protein Embp by osteoblasts. *S. epidermidis* strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures.

4.4.6 PIA plays a role in the association of *S. epidermidis* with osteoblasts

The polysaccharide intercellular adhesin (PIA) is reported to be responsible for the hemagglutinating activity of *S. epidermidis* (Rupp et al., 1999). Association of *S. epidermidis* with osteoblasts was affected by disruption of PIA production. An isogenic mutant of *S. epidermidis* strain O-47 deficient in PIA production showed 40% less association with osteoblasts when compared to the wild type strain (figure 4-7).

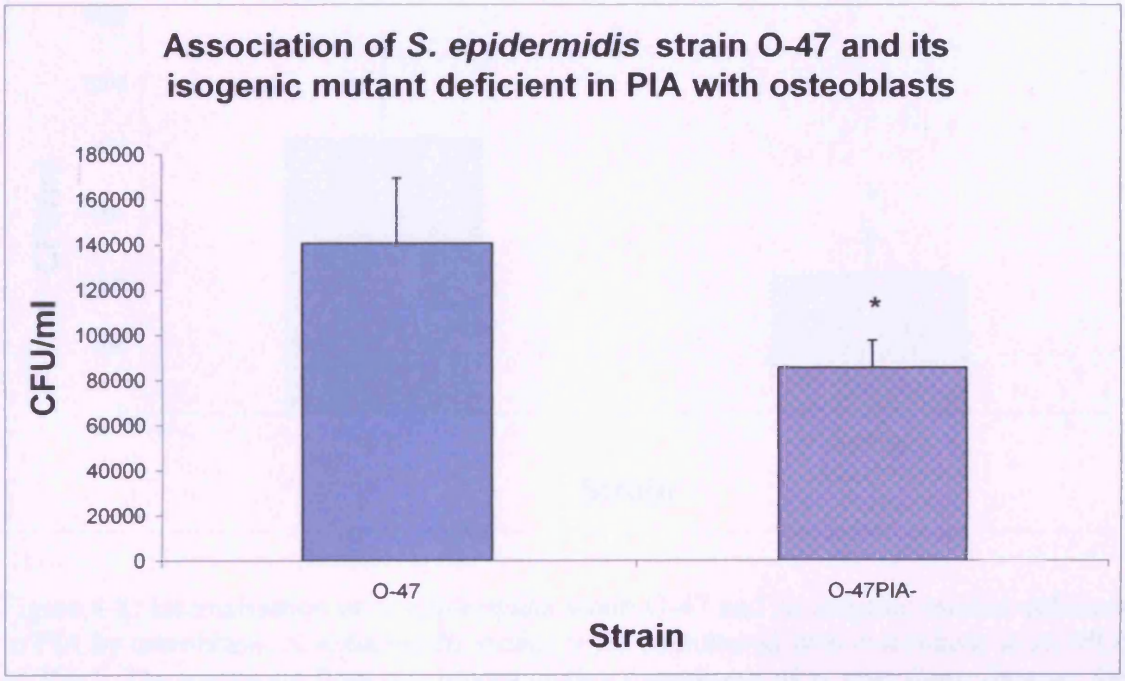

Figure 4-7: Association of *S. epidermidis* strain O-47 and its isogenic mutant deficient in PIA with osteoblasts. *S. epidermidis* strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. * P value < 0.05.

4.4.7 *S. epidermidis* PIA plays a role in the internalisation of strain O-47 by osteoblasts

S. epidermidis strain O-47 and its isogenic mutant with a Tn*917* insertion in the *ica* locus, that is deficient in PIA production (Rupp et al., 2001) were examined for their capacity to be internalised by osteoblasts. A *S. epidermidis* isogenic mutant of strain O-

47 deficient in PIA production showed a reduced capacity to be internalised by osteoblasts. Internalisation of the isogenic mutant was 50% of the parental strain (figure 4-8).

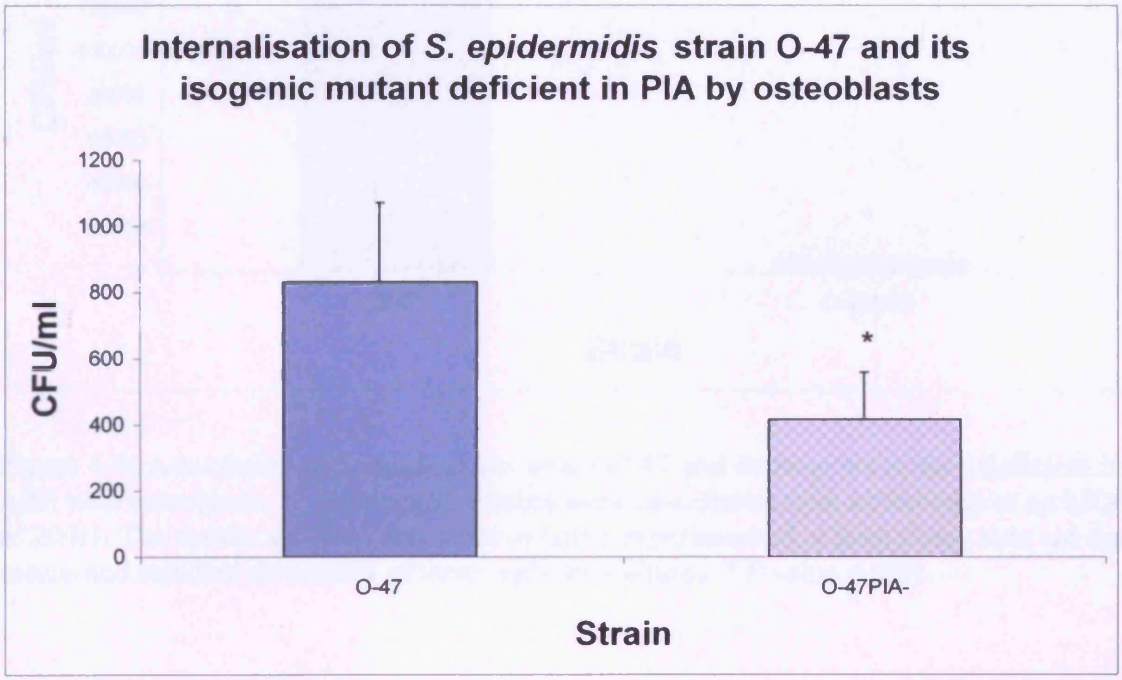

Figure 4-8: Internalisation of *S. epidermidis* strain O-47 and its isogenic mutant deficient in PIA by osteoblasts. *S. epidermidis* strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. * P value < 0.05.

4.4.8 The role of the autolysin AtlE in *S. epidermidis* association with osteoblasts

Association of *S. epidermidis* with osteoblasts was affected by disruption of the gene coding for AtlE. The *S. epidermidis* mutant strain deficient in AtlE showed 96% less association with osteoblasts when compared to the wild type strain (figure 4-9).

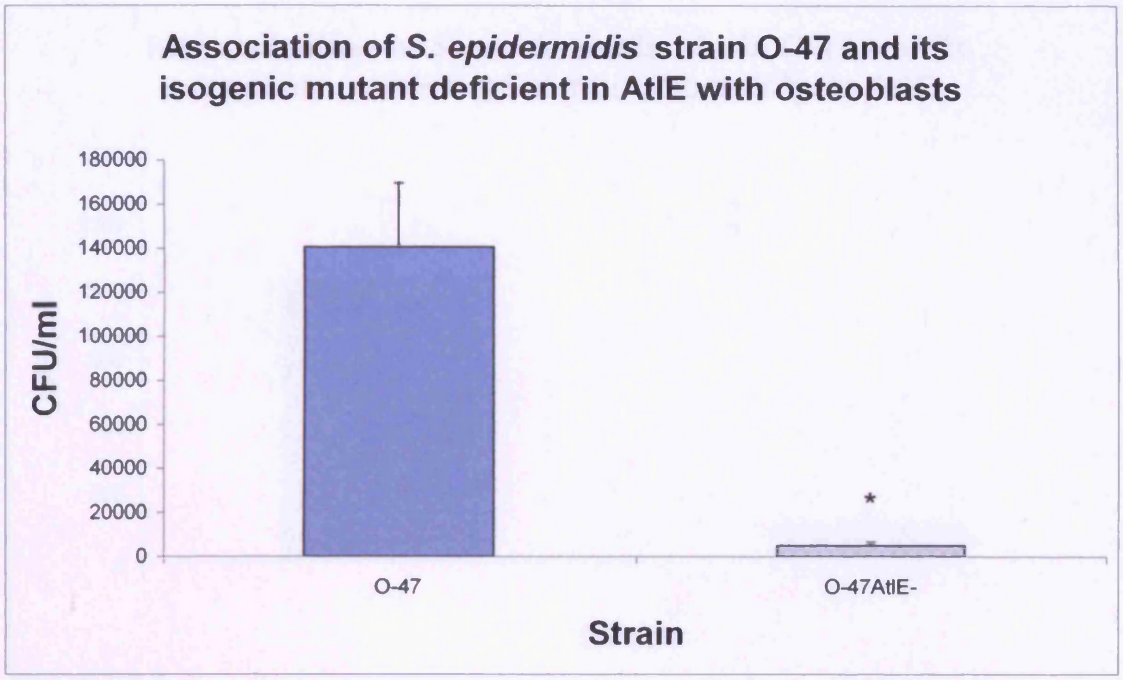

Figure 4-9: Association of *S. epidermidis* strain O-47 and its isogenic mutant deficient in AtlE with osteoblasts. *S. epidermidis* strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. * P value < 0.01.

4.4.9 *S. epidermidis* autolysin AtlE plays a role in the internalisation of *S. epidermidis* by osteoblasts

Autolysins have been found to be responsible for attachment of bacteria to plastics and eukaryotic cells (Milohanic et al., 2001). The role of the *S. epidermidis* autolysin AtlE in the internalisation of this bacterium by osteoblasts was investigated. *S. epidermidis* O-47 and its isogenic mutant with a Tn*917* insertional mutation in the gene coding for AtlE were used in this experiment. The level of internalisation of the mutant was compared to that of the wild type parental strain (figure 4-10). The AtlE mutant had a significantly reduced capacity to be internalised by osteoblasts.

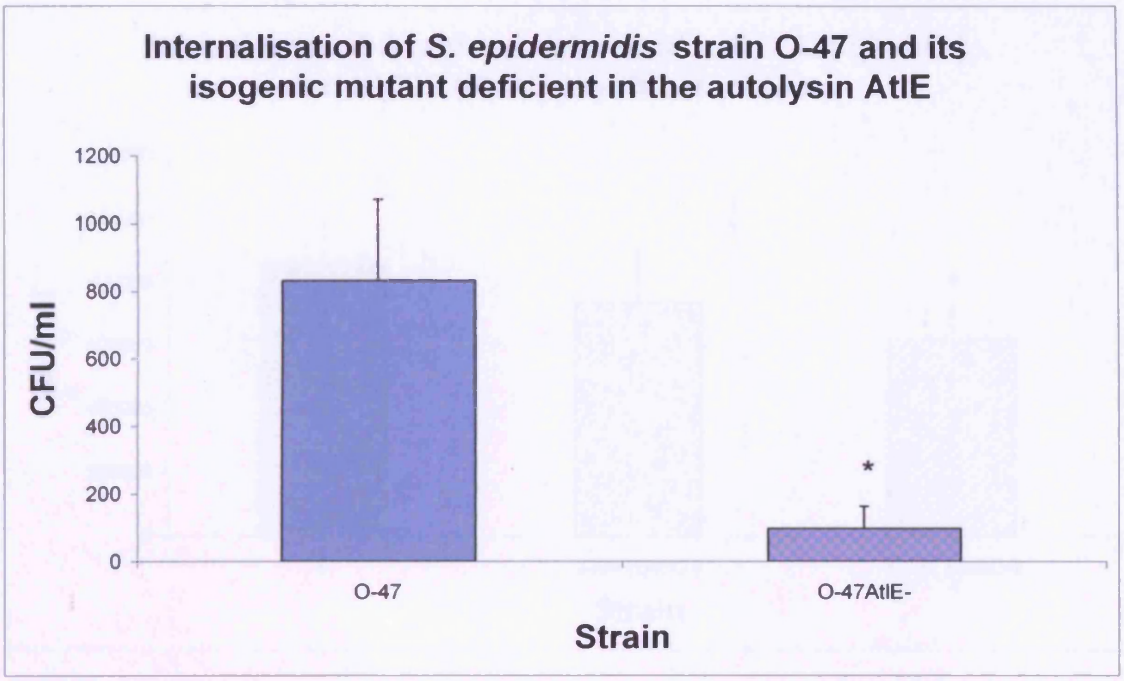

Figure 4-10: Internalisation of *S. epidermidis* strain O-47 and its isogenic mutant deficient in AtlE by osteoblasts. *S. epidermidis* strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. * P value < 0.01.

4.4.10 The GehD but not the GehC lipase is involved in the association of *S. epidermidis* with osteoblasts

The nucleotide sequences of nine lipase genes from six different staphylococcal species have been published. Three are derived from *S. epidermidis* (two from *S. epidermidis* 9 and one from *S. epidermidis* RP62A) (Rosenstein and Gotz, 2000). It has been postulated that the lipases are virulence factors of these organisms. To determine the role of *S. epidermidis* lipases in the association of this bacterium with osteoblasts two isogenic mutants of *S. epidermidis* deficient in GehC and GehD lipases were used. Mutation of the GehD gene resulted in a reduction in the association of *S. epidermidis* with osteoblasts by 28% (figure 4-11).

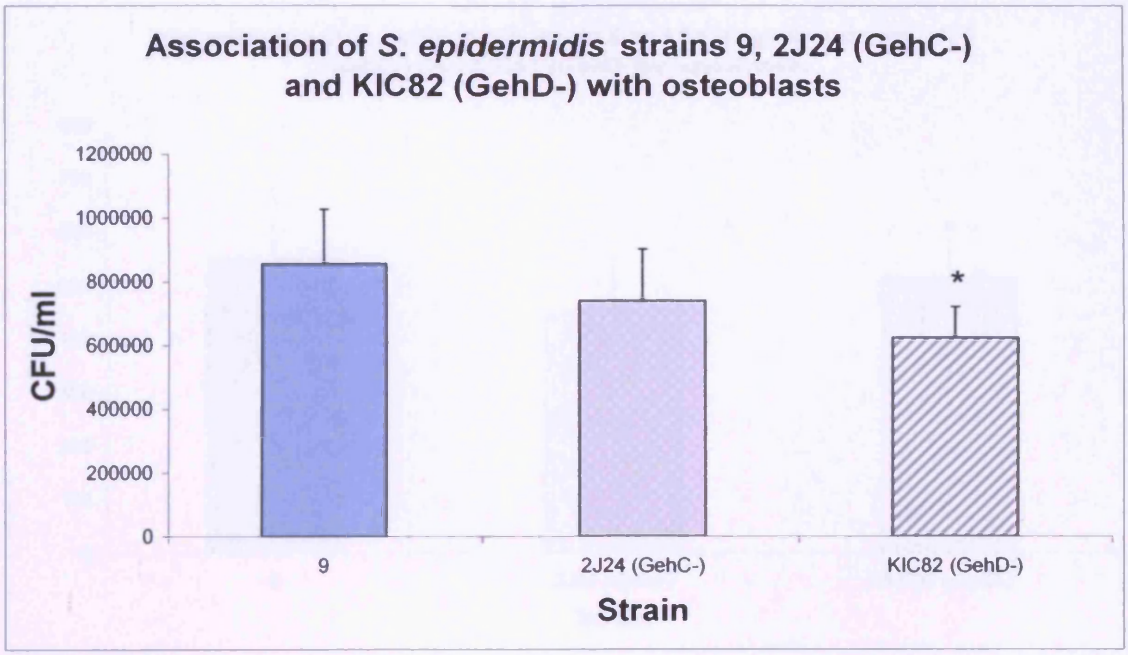

Figure 4-11: Association of *S. epidermidis* strains 9, 2J24 (GehC-), and KIC82 (GehD-) with osteoblasts. *S. epidermidis* strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. * P value < 0.05.

4.4.11 GehD and GehC lipase are not involved in internalisation of *S. epidermidis* by osteoblasts

To examine the role of the GehC and GehD lipases in the internalisation of *S. epidermidis* by osteoblasts, *S. epidermidis* mutants deficient in either GehD (KIC82) or GehC (2J24) were compared to the wild type parental strain in internalisation assays. Figure 4-12 shows that neither of the *S. epidermidis* lipases contributed significantly to the internalisation of *S. epidermidis* by osteoblasts.

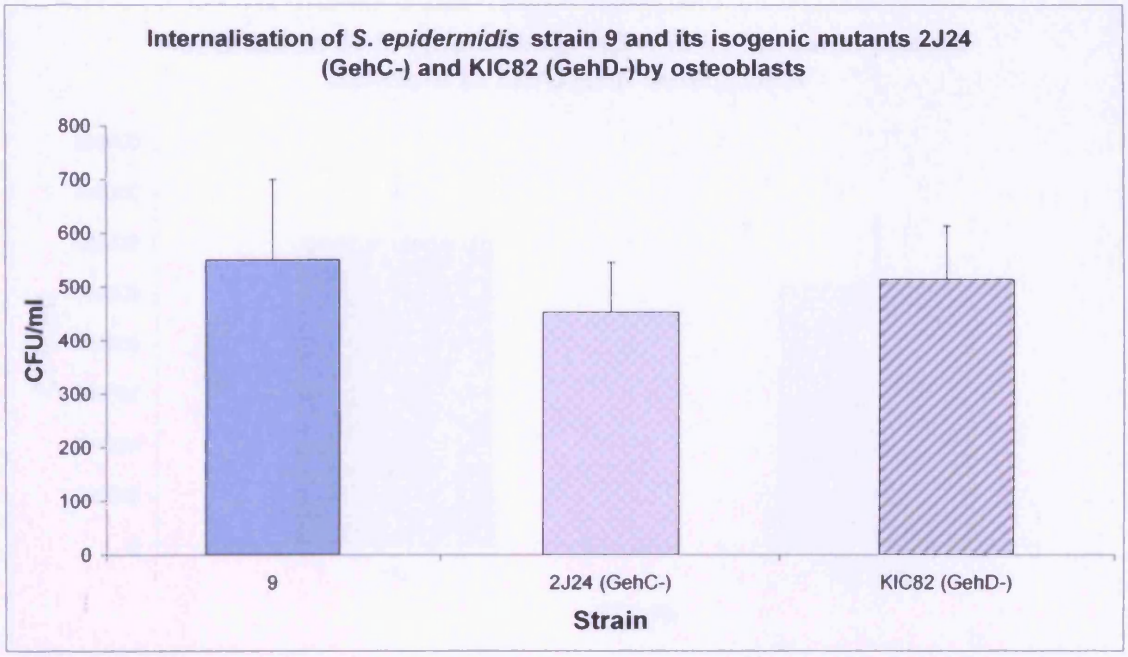

Figure 4-12: Internalisation of *S. epidermidis* strains 9, 2J24 (GehC-), and KIC82 (GehD-) by osteoblasts. *S. epidermidis* strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures.

4.4.12 SdrG is not involved in the association of *S. epidermidis* strain HB with osteoblasts

The SdrG of *S. epidermidis* is a surface protein that promotes adherence of bacteria to immobilised fibrinogen (Hartford et al., 2001). To examine the effect of *S. epidermidis* SdrG on the association of this bacterium with osteoblasts, an isogenic mutant of *S. epidermidis* strain HB deficient in SdrG was used in this experiment. There was no difference in the association of the mutant strain with osteoblasts when compared to the wild type parental strain (figure 4-13).

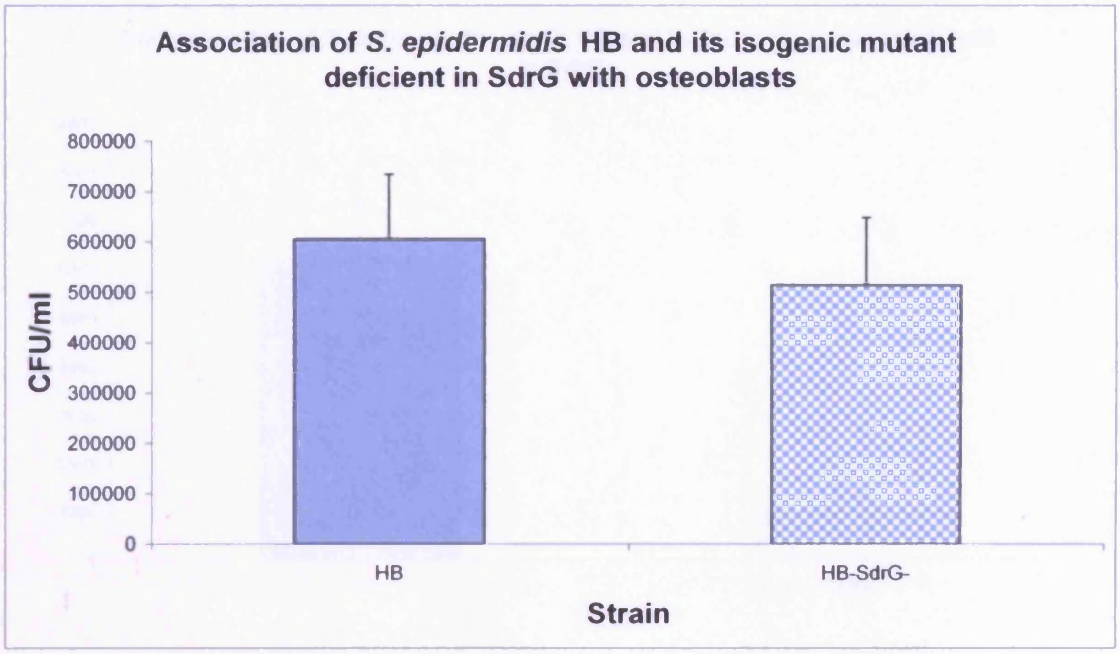

Figure 4-13: Association of *S. epidermidis* strain HB and its isogenic mutant deficient in SdrG with osteoblasts. Strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures.

4.4.13 SdrG plays an important role in the internalisation of *S. epidermidis* strain HB by osteoblasts

To determine if SdrG of *S. epidermidis* is involved in the internalisation of this organism by osteoblasts, *S. epidermidis* strain HB and its isogenic mutant deficient in SdrG were examined for their capacity to be internalised by osteoblasts. The SdrG deficient isogenic mutant of *S. epidermidis* showed a reduced level of internalisation when compared to the parental wild type strain (figure 4-14). These findings indicate that SdrG of *S. epidermidis* has a role in the internalisation of *S. epidermidis* strain HB by osteoblasts.

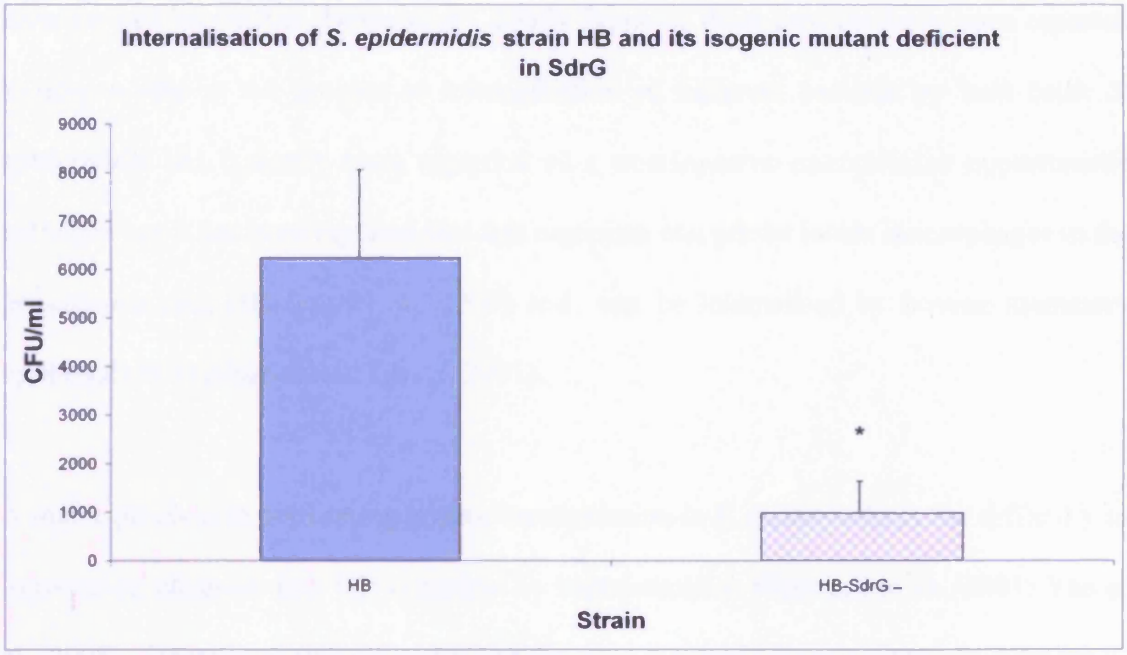

Figure 4-14: Internalisation of *S. epidermidis* strain HB and its isogenic mutant deficient in SdrG by osteoblasts. Strains were co-cultured with osteoblasts at an MOI of 200:1. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. * P value < 0.01.

4.5 Discussion

In the experiments presented in chapter 3 a panel of inhibitors were used to determine if internalisation of *S. epidermidis* by osteoblasts was a receptor mediated process. In this chapter, data examining the role of different *S. epidermidis* virulence factors in the internalisation of *S. epidermidis* by osteoblasts are presented. The establishment of bone infection by bacteria depends on the ability of the organism to evade host defense systems. This can be by the production of extracellular proteins and toxins or enzymes such as lipases and esterases which can have an impact on specific host targets or by forming biofilms (Vuong and Otto, 2002). Examples of these proteins are host cell proteins such as E-cadherins and integrins or bacterial proteins such as

invasins and internalins (Isberg et al., 2000). Some of these proteins have been reported to play a role in the process of internalisation of different bacteria by host cells. *S. epidermidis* has typically been regarded as a non-invasive extracellular opportunistic pathogen but it has been reported that this organism can persist inside macrophages in the pericatheter area (Boelens et al., 2000) and can be internalised by bovine mammary epithelial cells (Almeida and Oliver, 2001).

A major problem in performing genetic manipulation in *S. epidermidis* is the difficulty in introducing plasmids into this organism by transformation (Hartford et al., 2001; Yao et al., 2005). Because of this difficulty and the time it would take to construct a number of isogenic mutants in a single strain we utilised strains of *S. epidermidis* that were publicly available and for which isogenic mutants of *S. epidermidis* virulence determinants were available to examine their potential role in the process of internalisation by host cells. Internalisation of *S. aureus* by osteoblasts has been proposed to occur through a bridge involving the bacterial fibronectin binding proteins, fibronectin and the host cell integrin $\alpha5\beta1$ (Alexander and Hudson, 2001). Ahmed *et al* have shown that disruption of the *S. aureus* fibronectin binding proteins (FnBPA and FnBPB) can block internalisation of *S. aureus* by osteoblasts but adhesion to osteoblasts is not affected (Ahmed et al., 2001). The use of rFnBPB[D1-D4] has been shown to inhibit internalisation of *S. aureus* by osteoblasts (Ahmed et al., 2001). To examine if *S. epidermidis* expresses a similar protein that may be involved in the internalisation process, a recombinant fragment of the *S. aureus* FnBPB[D1-D4] was added to osteoblasts one hour before adding *S. epidermidis*. The *S. aureus* rFnBPB[D1-D4] had no significant effect on the internalisation of *S. epidermidis* by osteoblasts (figure 4-3). The effect of rFnBPB[D1-D4] on the association

of *S. epidermidis* with osteoblasts was also investigated. There was no inhibitory effect of this recombinant protein on *S. epidermidis* association with osteoblasts (figure 4-2). These findings suggest that *S. epidermidis* did not express a protein with similar binding properties to that of *S. aureus*. Williams et al, have recently identified a fibronectin binding protein, Embp of *S. epidermidis* which seems to be distinct from the *S. aureus* fibronectin binding protein (Williams et al., 2002). A recombinant protein Embp32, which encompassed the fibronectin binding domain of this protein, has been shown to block the capacity of this bacterium to bind to fibronectin but has no effect on binding of *S. aureus* (Williams et al., 2002). It has been shown that the fibronectin binding proteins of *S. aureus* are essential for both internalisation and adhesion of this bacterium to bovine mammary gland cells (Lammers et al., 1999). Peacock *et al* have shown that the fibronectin binding proteins of *S. aureus* can mediate adhesion to endothelial cells and are required for subsequent internalisation (Peacock et al., 1999). Integrins are heterodimers that mediate communication and adhesion between cells and the extracellular matrix. The interaction between *S. aureus* and host cells was proposed to be through a bridge where fibronectin which was bound to the *S. aureus* fibronectin binding proteins interacted with the host cell integrin α5β1 (Alexander and Hudson, 2001). *S. epidermidis* strains that are disrupted in the fibronectin binding protein Embp did not show a decreased capacity to be internalised by osteoblasts (figure 4-6). To examine whether *S. epidermidis* internalisation depends on an interaction with the host cell integrin α5β1, blocking antibodies against this integrin were used to inhibit its binding activity on the surface of osteoblasts. The use of the blocking antibody in the internalisation assay did not inhibit internalisation of *S. epidermidis* by osteoblasts (figure 4-6). The finding that *S. aureus* FnBPs could not block the internalisation of *S.*

epidermidis and that neither the *S. epidermidis* fibronectin binding protein Embp nor host cell integrin α5β1 are required for internalisation of this bacterium by osteoblasts highlights that this organism gains access to the host cells via a mechanism that is different from that utilised in the uptake of *S. aureus*.

S. epidermidis mutants deficient in either polysaccharide intercellular adhesin or the autolysin AtlE showed a significant reduction in their capacity to be internalised by osteoblasts. Internalisation of *S. epidermidis* strains deficient in PIA or AtlE was reduced by 50% and 88% respectively (figures 4-8 & 4-10). There was also a decrease in association of the mutant strains deficient in PIA or AtlE with osteoblasts (reduced by 40% and 96% respectively) (figures 4-7 & 4-9). These findings indicate that reduction in the capacities of these strains to be internalised was probably due to the reduced capacity to bind to osteoblasts. *S. epidermidis* has two lipases GehC and GehD which have been suggested to be virulence determinants and could be involved in pathogenesis of diseases caused by this organism. It has been reported that the GehD lipase is a bifunctional molecule that also acts as a collagen adhesin (Bowden et al., 2002). *S. epidermidis* lipases have been reported to be important in skin colonisation (Bowden et al., 2002). The effect of the two lipases of *S. epidermidis* on the internalisation of this bacterium by osteoblasts was examined. Internalisation of *S. epidermidis* isogenic mutants deficient in either GehD or GehC lipases by osteoblasts was similar to the parental strain (figure 4-12). On the other hand disruption of gene for the GehD lipase reduced *S. epidermidis* association with osteoblasts (figure 4-11). These findings suggest that lipases are not involved in internalisation of *S. epidermidis* by osteoblasts but that GehD lipase plays a role in the association of this bacterium with osteoblasts.

Similar to *S. aureus*, *S. epidermidis* has been found to express a fibrinogen binding protein (Nilsson et al., 1998). Fbe is 119-kDa protein located on the surface of *S. epidermidis* and is present in most strains of *S. epidermidis* (Nilsson et al., 1998). This protein is structurally related to *S. aureus* clumping factor A and was also named SdrG (Pei et al., 1999). Antibodies against SdrG can enhance phagocytosis of *S. epidermidis* by macrophages and decrease the severity of infection caused by this organism (Rennermalm et al., 2004). Investigation of the internalisation of a *S. epidermidis* mutant deficient in SdrG by osteoblasts has shown that SdrG is essential for the internalisation of *S. epidermidis* strain HB (figure 4-14). The internalisation of the SdrG deficient strain by osteoblasts was impaired by 84% when compared to the internalisation of the wild type strain (figure 4-14). Recently it has been shown that the fibrinogen binding protein of *Streptococcus agalactiae* is essential in the internalisation of this bacterium by epithelial cells (Gutekunst et al., 2004). Disruption of the gene for the SdrG protein of *S. epidermidis* did not significantly affect adhesion of this organism to osteoblasts (figure 4-13). The data presented in chapter 3 demonstrated that different strains of *S. epidermidis* are internalised by osteoblasts through different pathways. Cytochalasin D increased the internalisation of *S. epidermidis* strain 19 by osteoblasts while inhibiting that of strains HB, 9 and O-47. This indicates that *S. epidermidis* strain 19 uses a different pathway in gaining access to osteoblasts than that utilised by other strains such as strains HB, 9 and O-47. Whether SdrG is also important in the internalisation of other strains of *S. epidermidis* by osteoblasts still needs to be investigated. This could be done by either constructing isogenic mutants of those strains or the use specific antibodies against SdrG.

4.6 Conclusions

Collectively, the results presented in this chapter suggest that some of the *S. epidermidis* virulence factors examined herein are involved in the internalisation of this bacterium by osteoblasts. The SdrG protein of *S. epidermidis* seems to play an important role in the internalisation of at least *S. epidermidis* strain HB by osteoblasts. *S. epidermidis* mutants deficient in PIA or the autolysin AtlE had reduced capacities to be internalised and to associate with osteoblasts. The fibronectin binding protein Embp, the GehD lipase and GehC lipase do not seem to play an important role in the internalisation of *S. epidermidis* by osteoblasts. Although there is little research on the factors that contribute to infections caused by *S. epidermidis*, these findings may add to our future understanding of how this bacterium causes disease.

Chapter 5

Chapter 5

Fate of *S. epidermidis* and osteoblasts following internalisation

5.1 Introduction

The immune response against an intracellular bacterial pathogen is complex and is influenced by the expression of numerous signalling molecules, including cytokines. It has previously been shown that secretion of interleukin (IL)-12 by macrophages or dentritic cells is critical for optimal cell-mediated immune responses against a variety of intracellular pathogens (Koblish et al., 1998). The production of other cytokines, such as IL-6, can further augment the lymphocyte and macrophage response. Although the major function of the osteoblast is to synthesise the components of bone matrix, mainly type one collagen and to catalyse mineralisation of the matrix (Mackie, 2003) , they have the potential to secret cytokines such as IL-1, IL-6, tumor necrosis factor alpha, and IL-8 (Armour et al., 1999; Rifas, 1999; van't Hof and Ralston, 2001). The production of these cytokines has been associated with the ability of osteoblasts to modulate the action of osteoclasts and promote bone resorption (Gonzalez, 2000). Infecting osteoblasts with *S. aureus* induces the production of high levels of IL-6 and IL-12 (Bost et al., 1999). The intracellular survival of the bacterium in osteoblasts and the subsequent induction of proinflamatory cytokines may be involved in bone infection (Alexander et al., 2001).

It has been reported that some bacteria can induce apoptosis after they are internalised by host cells. Metabolically active *S. aureus* has been shown to induce apoptosis in different cell types such as keratinocytes, osteoblasts and epithelial cells (Menzies and Kourteva, 1998; Alexander and Hudson, 2001). In this study, we investigated the modulation of

cytokine production in response to internalisation of *S. epidermidis* by osteoblasts and the fate of the bacteria and the osteoblast subsequent to uptake.

5.2 Materials and Methods

5.2.1 Internalisation assay

Internalisation assays were carried out as described in chapter 2, section 2.5.2.

5.2.2 Cytokine production by osteoblasts in response to internalisation of *S. epidermidis*

Detection of four different cytokines (IL-6, IL-8, IL-1β and TNF-α) was performed using ELISAs as described in chapter 2, section 2.5.2.1.

5.2.3 Trypan blue exclusion assay

Osteoblasts were infected with *S. epidermidis* or *S. aureus* for various times. Infected and non-infected (control) osteoblasts were washed, trypsinized and mixed with an equal volume of trypan blue (0.5%, w/v, in PBS) (Sigma, UK). A small amount of the mixture (10 µl) was placed on a hemocytometer, and the numbers of total and stained cells were determined under a light microscope. The percentage of damaged osteoblasts was calculated by dividing the mean number of damaged (stained) cells by the total number of cells multiplied by 100.

5.2.4 Assessment of osteoblast apoptosis

Translocation of phosphatidylserine to the outer layer of the cell membrane is an early feature of apoptosis. The presence of exposed phosphatidylserine on the surface of osteoblasts was detected with Annexin-V-FLUOS (Molecular Probes, UK). Osteoblasts grown in a 24-well plate were infected either with *S. epidermidis* or *S. aureus* at an MOI of 10:1 for 2 h. The culture medium was then replaced with fresh medium supplemented with gentamicin (50 µg/ml), and incubation proceeded at 37° C. At 16 hours post internalisation, cells were washed with PBS and resuspended in labeling solution containing Annexin-V-FLOUS. Incubation buffer, containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 5 mM $CaCl_2$, was added to each sample and the phosphatidylserine was detected with a FACScan flow cytometer (Becton Dickinson) using a FITC signal detector. Data were recorded on a logarithmic scale and processed with specific Cell Quest software (Becton Dickinson) for FACScan research.

5.3 Statistics

All data are shown as the mean ± the standard deviation. Data were compared using Student's t-test which allows mean comparisons in small samples. In some experiments a Mann Whitney test was used to assess the significance.

5.4 Results

5.4.1 Production of IL-6 in response to internalisation of *S. epidermidis* by osteoblasts

Cytokine production by osteoblasts was monitored over time using ELISA assay. Two *S. epidermidis* strains were used in this experiment, strain 19, which has been shown to be highly invasive (chapter 3) and strain NCTC11964, which was found to be less invasive of osteoblasts (chapter3).

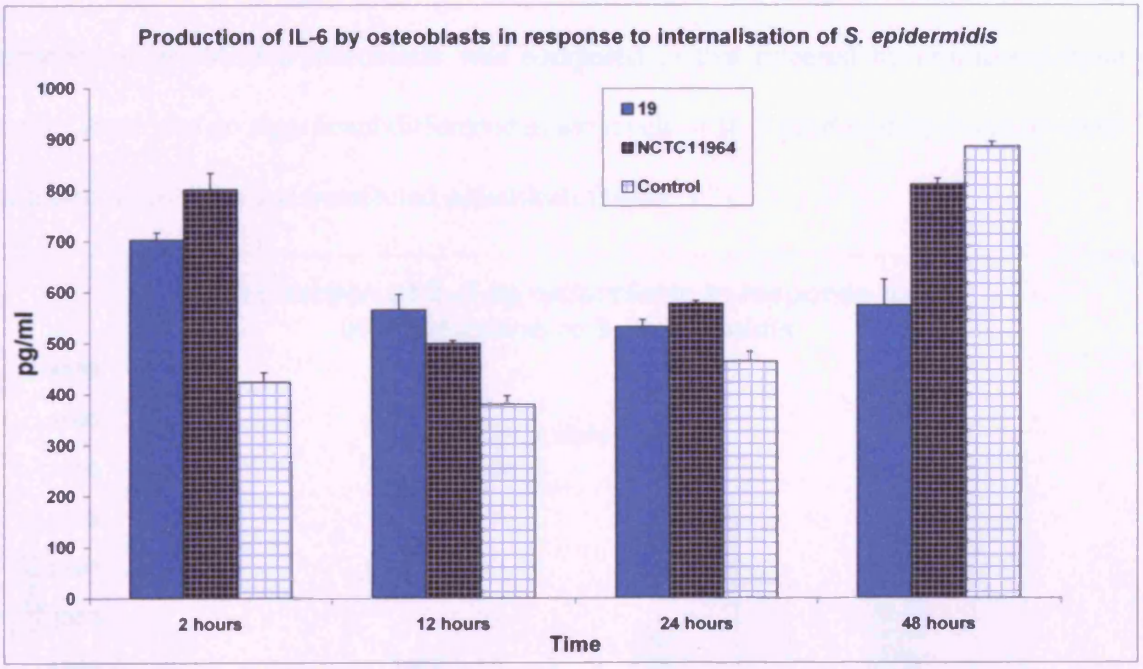

Figure 5-1: Production of IL-6 by osteoblasts in the presence or absence of *S. epidermidis* strains 19 and NCTC11964. Data are the mean and standard deviation of three replicate cultures. The IL-6 levels produced by *S. epidermidis* infected osteoblasts were compared to those produced by uninfected osteoblasts.

Cytokine production by infected osteoblasts was compared to that released by uninfected bone cells. There was a significant (P <0.05) difference in the production of IL-6 by osteoblasts after a two hour internalisation period for both strains. However over a longer

time period of 12 to 48 hours there was no significant difference in the level of IL-6 produced by *S. epidermidis* infected osteoblasts and uninfected osteoblasts (figure 5-1). The levels of IL-6 produced by osteoblasts whether infected or not were relatively low.

5.4.2 Production of IL-8 by osteoblasts in response to internalisation of *S. epidermidis*

Production of IL-8 by osteoblasts was monitored over time in the presence or absence of *S. epidermidis* strains 19 and NCTC11964 using ELISA assay. IL-8 production by infected osteoblasts was compared to that released by uninfected bone cells. There was no significant difference in the levels of IL-8 produced by *S. epidermidis* infected osteoblasts and uninfected osteoblasts (figure 5-2).

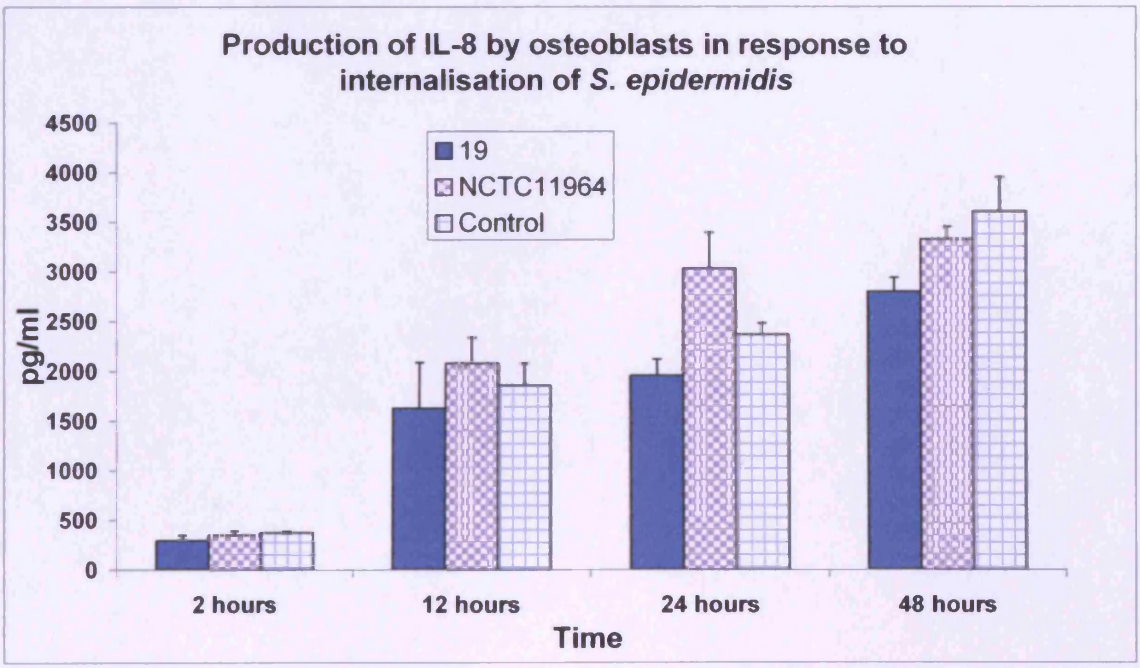

Figure 5-2: Production of IL-8 by osteoblasts in the presence or absence of *S. epidermidis* strains 19 and NCTC11964. Data are the mean and standard deviation of three replicate cultures. The IL-8 levels produced by *S. epidermidis* infected osteoblasts were compared to those produced by uninfected osteoblasts.

5.4.3 Production of IL-1β by osteoblasts in response to internalisation of *S. epidermidis*

Using ELISA we have examined the level of IL-1β produced by osteoblasts in the presence or absence of *S. epidermidis* strains 19 and NCTC11964. The results were compared to the level of IL-1β produced by osteoblasts in the presence of no bacteria. The results show that there was no significant difference in the levels of IL-1β produced by osteoblasts in the presence or absence of *S. epidermidis* (figure 5-3).

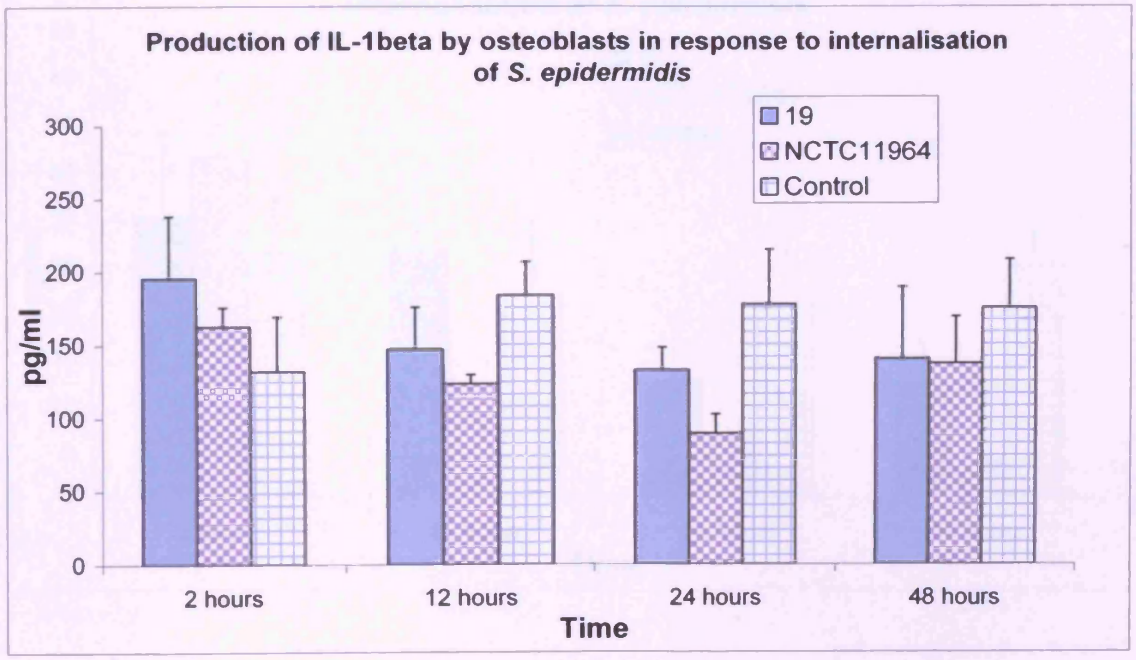

Figure 5-3: Production of IL-1β by osteoblasts in the presence or absence of *S. epidermidis* strains 19 and NCTC11964. Data are the mean and standard deviation of three replicate cultures. The IL-1β levels produced by *S. epidermidis* infected osteoblasts were compared to those produced by uninfected osteoblasts.

5.4.4 Production of TNF-α in response to internalisation of *S. epidermidis*

Production of TNF-α by osteoblasts was monitored over time using ELISA assay. The production of TNF-α by *S. epidermidis* infected osteoblasts was compared to that released by uninfected osteoblasts. The results show that there was no significant difference in the levels of TNF-α produced by osteoblasts in the presence or absence of *S. epidermidis* at 2, 12, 24 and 48 hours (figure 5-4).

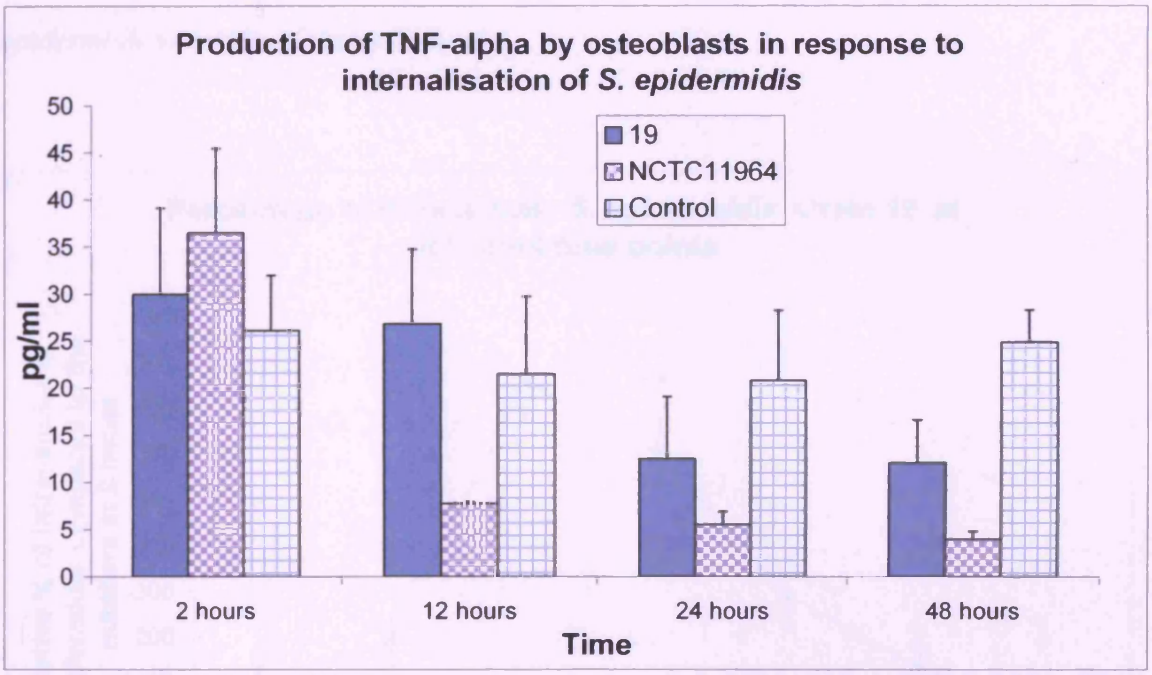

Figure 5-4: Production of TNF-α by osteoblasts in the presence or absence of *S. epidermidis* strains 19 and NCTC11964. Data are the mean and standard deviation of three replicate cultures. The TNF-α levels produced by *S. epidermidis* infected osteoblasts were compared to that produced by uninfected bone cells.

5.4.5 Fate of *S. epidermidis* following internalisation by osteoblasts

The internalisation assay was performed as described in chapter 2, section 2.5.2. Osteoblasts were washed three times with PBS and intracellular *S. epidermidis* that remained viable inside osteoblasts incubated in the presence of gentamicin containing medium were recovered by lysis of the osteoblasts at different time points. There was a significant increase in the numbers of intracellular *S. epidermidis* at 12 hours post infection. After 24 hours the numbers of intracellular *S. epidermidis* began to decline with time (figure 5-5). At 96 hours post internalisation the percentage of intracellular *S. epidermidis* was 41% of that at 2 hours.

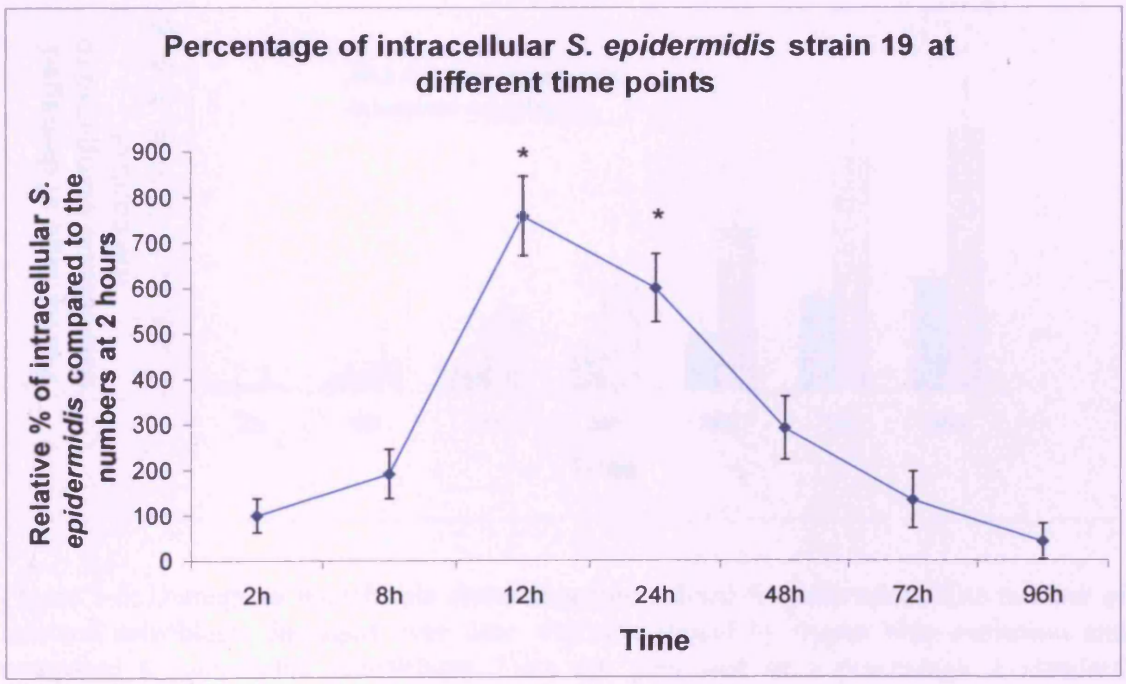

Figure 5-5: Growth of intracellular *S. epidermidis* strain 19. The number of intracellular *S. epidermidis* in osteoblasts was determined at different time points. Data are presented as a relative percentage compared to the numbers internalised at 2 hours ± standard deviation. The experiment is a representative of at least 3 experiments. * P value < 0.05.

5.4.6 Damage of osteoblasts by internalised *S. epidermidis*

Damage of osteoblasts containing internalised *S. epidermidis* was examined at various time points after a standard internalisation assay. The medium covering the osteoblasts contained 50 µg/ml gentamicin. Figure 5-6 shows that there was a significant difference over time in the numbers of infected osteoblasts that were damaged, as determined with the trypan blue exclusion assay, compared to infected osteoblasts (figure 5-6). At 96 hours post infection 20% more infected osteoblasts appeared to be damaged compared to uninfected osteoblasts (figure 5-6).

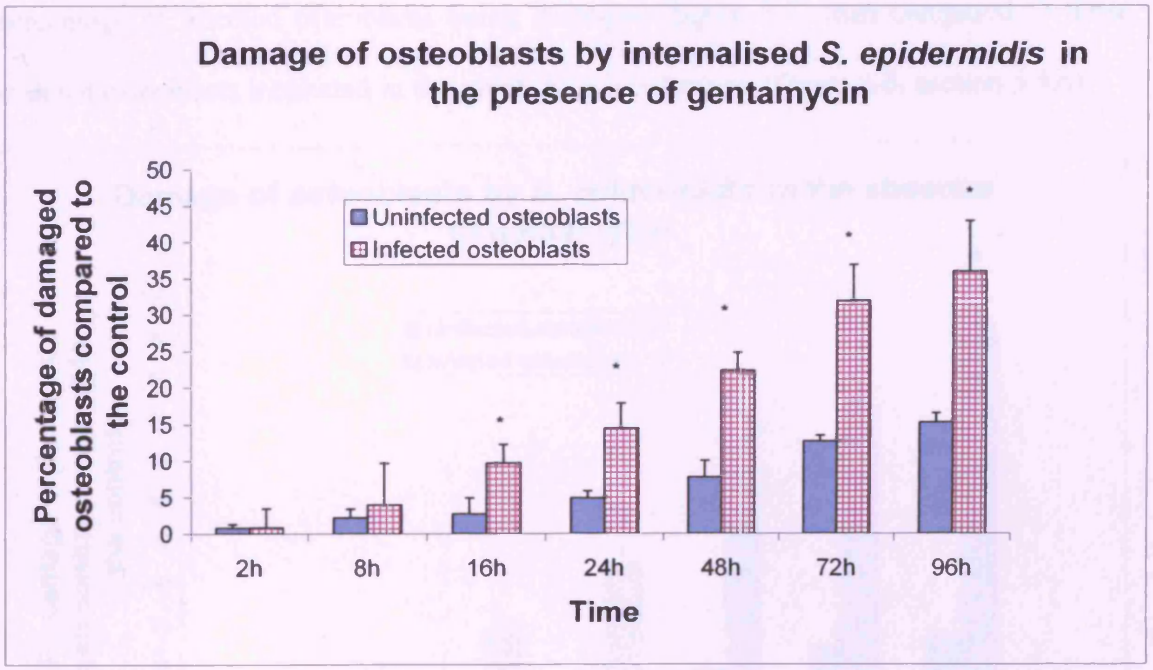

Figure 5-6: Damage of osteoblasts containing internalised *S. epidermidis*. The number of infected osteoblasts damaged over time was determined by trypan blue exclusion and compared to uninfected osteoblasts. Data are presented as a percentage ± standard deviation. The experiment is a representative of at least 3 experiments. * P value < 0.05.

5.4.7 Damage of osteoblasts by internalised *S. epidermidis* in the absence of gentamicin

In the absence of gentamicin in the assay medium there was a significant increase in the number of infected osteoblasts damaged compared to the uninfected osteoblasts after 16 hours (figure 5-7). The numbers of infected osteoblasts damaged increased with time until the end point of the assay at 96 hours. At the 96 hour time point 90% ($P < 0.01$) of the infected osteoblasts were damaged compared to 15% of the uninfected osteoblasts. The absence of gentamicin in the assay medium led to a significantly higher percentage of infected osteoblasts being damaged (figure 5-7) than compared to those infected osteoblasts incubated in the presence of gentamicin (figure 5-6, section 5.4.6)

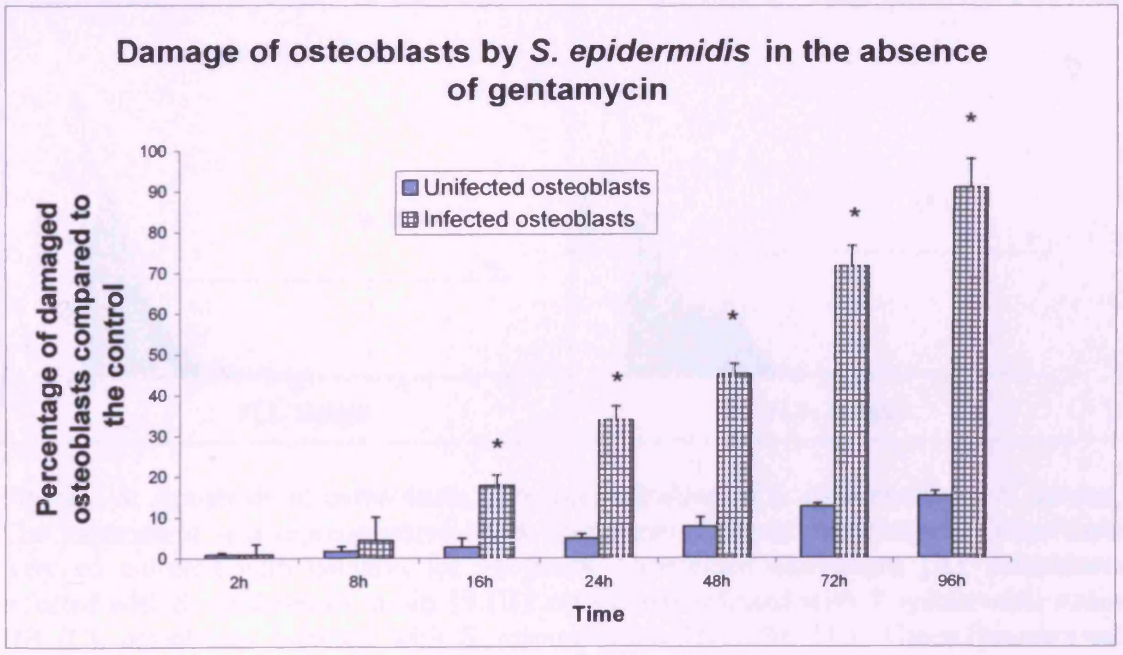

Figure 5-7: Damage of osteoblasts by internalised *S. epidermidis* in the absence of gentamicin. The number of infected osteoblasts damaged by internalised *S. epidermidis* was determined by trypan blue exclusion and compared to uninfected osteoblasts. Data are presented as a percentage ± standard deviation. The experiment is a representative of at least 3 experiments. * P value < 0.05.

5.4.8 Apoptosis of osteoblasts in response to internalised *S. epidermidis* or *S. aureus*

Bacteria are able to trigger apoptosis of mammalian cells by a variety of mechanisms including the secretion of protein synthesis inhibitors, pore forming proteins and activation of the host cells endogenous death machinery (Grassme et al., 2001).

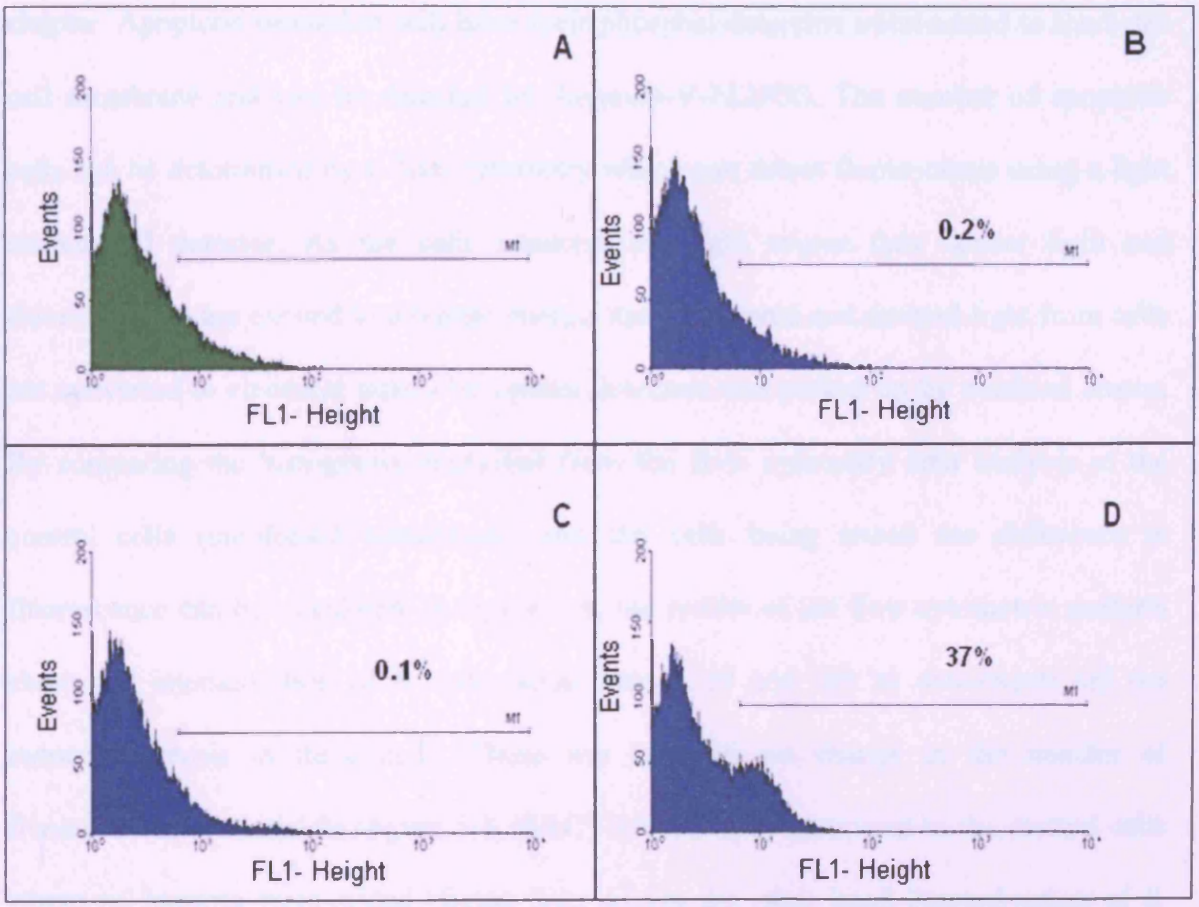

Figure 5-8: Apoptosis of osteoblasts after internalisation of *S. epidermidis* or *S. aureus*. The experiment is a representative of at least three different experiments. Osteoblasts were co cultured with bacteria for 16 hours. Uninfected osteoblasts (A), osteoblasts infected with *S. epidermidis* strain 19 (B), osteoblasts infected with *S. epidermidis* strain HB (C), osteoblasts infected with *S. aureus* strain LS1 (D). FL1, Green fluorescence signal received by the photomultiplier tube. M1, percentage of fluorescence change compared to the control cells (uninfected cells).

In this experiment we examined the ability of *S. epidermidis* to induce apoptosis in osteoblasts following internalisation. Three strains were examined for their capacity to induce apoptosis of osteoblasts. Two strains of *S. epidermidis* (strain 19 and HB) were

selected because of their different capacities to be internalised by osteoblasts. *S. aureus* strain LS-1 which has been shown to have a high capacity to be internalised by osteoblasts and induce apoptosis was used as a positive control (Menzies and Kourteva, 1998). Apoptosis was detected by apoptosis kit as described in section 5.2.4 in this chapter. Apoptotic osteoblast will have their phosphatidylserine translocated to the outer cell membrane and can be detected by Annexin-V-FLUOS. The number of apoptotic cells can be determined by a flow cytometry which can detect fluorescence using a light source and detector. As the cells intercept the light source they scatter light and fluorochromes are excited to a higher energy state. Scattered and emitted light from cells are converted to electrical pulses by optical detectors and picked up by confocal lenses. By comparing the histograms generated from the flow cytometry data analysis of the control cells (uninfected osteoblasts) and the cells being tested the difference in fluorescence can be calculated. In figure 5-8, the results of the flow cytometric analysis show that internalisation of *S. epidermidis* strains 19 and HB by osteoblasts did not induce apoptosis in these cells. There was insignificant change in the number of fluorescent *S. epidermidis* (figure 5-8, B&C) infected cells compared to the control cells where no bacteria were added (figure 5-8, A) On the other hand internalisation of *S. aureus* by osteoblasts induced apoptosis (figure 5-8, D) as seen by the increase ($P < 0.01$) in fluorescence detected from infected osteoblasts by flow cytometry compared to the fluorescence from uninfected osteoblasts.

5.5 Discussion

The immune system response to intracellular bacteria is complex and influenced by the expression of numerous signalling molecules, including cytokines. It has previously been shown that secretion of IL-12 by macrophages or dendritic cells is critical for the optimal cell mediated immune response against a variety of intracellular pathogens, such as *Salmonella* species (Kincy-Cain et al., 1996). It has been shown that levels of some cytokines such as TNF-α and IL-6 in blood are elevated in patients with sepsis and appear to be good markers for diagnosis of bacterial infection (Wang et al., 2000). It has been established during the past few years that cytokines such as IL-1, IL-6, IL-11, and TNF-α can stimulate osteoclast development and thereby the process of bone resorption (Hill, 1998; Xing and Boyce, 2005). Moreover, upregulation of the production and/or action of IL-6 has been implicated in the pathogenesis of disease states characterised by excessive osteoclastic bone resorption (Ralston and Grabowski, 1996; Gonzalez, 2000). We have examined whether internalisation of *S. epidermidis* by osteoblasts has any effect on the production of the cytokines IL-6, IL-8, IL-1β and TNF-α by osteoblasts. To examine the difference in cytokine induction by different strains, two strains of *S. epidermidis* with different capacities to be internalised by osteoblasts were used. Neither strain induced cytokine production by osteoblasts. The kinetics of cytokine expression by endothelial cells following infection with different strains of *S. aureus* has been shown to vary. For example it has been shown that the laboratory strain 8325-4 failed to induce either IL-1β or IL-6 in endothelial cells (Yao et al., 1995).

The consequences of intracellular *S. epidermidis* has not been investigated yet. The fate of *S. epidermidis* and osteoblasts following internalisation was examined in the

experiments detailed in this chapter. In the presence of gentamicin the intercellular *S. epidermidis* remained viable for up to 96 hours post infection which was the longest period examined in these experiments. The number of intracellular *S. epidermidis* started to increase over the 12 hours post infection then declined later on. This increase in intracellular bacteria suggests intracellular replication of *S. epidermidis*. Many bacteria such as *S. aureus* (Qazi et al., 2004) and *Neisseria meningitis* (Larson et al., 2002) have been show to replicate inside host cells. Osteoblast damage in the presence of *S. epidermidis* occurred whether or not antibiotic was present in the medium, but it was more prominent where no antibiotic was added at the post infection phase.

Induction of host cell apoptosis has been viewed as a virulence factor especially when the induced apoptosis is related to immune cells (Ross and Caligiuri, 1997). We found clear difference between *S. epidermidis* and *S. aureus* with regard to their ability to induce apoptosis of bone cell. It has been reported that *S. aureus* but not *S. epidermidis* can induce apoptosis in human neutrophils. This has been suggested to be related to the ability of *S. epidermidis* to consume intracellularly formed hydrogen peroxide which represents a key factor in many types of cell damage (Nilsdotter-Augustinsson et al., 2004). These findings suggest that one mechanism whereby *S. epidermidis* may cause sub-acute or chrronic infections is related to an inability to induce production of cytokines and apoptosis in bone cells.

5.6 Conclusions

The results described in this chapter showed that there were no significant levels of IL-6, IL-8, IL-1β and TNF-α produced by osteoblasts in the presence of intracellular *S. epidermidis*. *S. epidermidis* was able to replicate inside osteoblasts and able to escape from within bone cells. Although the presence of intracellular *S. epidermidis* caused some damage to the osteoblasts, apoptosis of bone cells was not induced by this bacterium. The ability of *S. epidermidis* to survive and replicate inside osteoblasts and its inability to induce cytokine production or cause apoptosis may be one of the explanations as to why this bacterium causes sub-acute or chronic infections.

Chapter 6

Chapter 6

Internalisation of *S. epidermidis* by epithelial cells

6.1 Introduction

The epithelium is an avascular tissue made of cells that cover exterior surfaces and line both internal closed cavities and body tubes that communicate with the exterior. Epithelium also forms the secretory portion of glands and ducts. It is considered the primary barrier of the body against microorganisms and plays an important role during surgical wound healing. Impaired surgical wound healing due to any factors such as bacterial infection can affect the outcome of the surgical procedure. Therefore it is important to study the reasons why some wounds become chronic and very difficult to deal with. The pathogenesis of *S. epidermidis* device related infections and the chronic nature of infections caused by this bacterium are poorly understood. Many reports have described the ability of different bacteria to be internalised by epithelial cells (Dziewanowska et al., 1999; Almeida et al., 1999; Almeida and Oliver, 2001; Evans et al., 2002; Kuo and Wang, 2003). This ability to invade epithelial cells has been shown as a key determinant of virulence for several human pathogenic bacteria such as *Salmonella* species (Huang et al., 1998a).

S. epidermidis has been shown to be internalised by bovine mammary epithelial cells (Almeida and Oliver, 2001). In chapter 3 osteoblasts were able to internalise *S. epidermidis* through a receptor mediated pathway. In this chapter the ability of epithelial cells to internalise *S. epidermidis* and the role of some putative virulence factors of this bacterium in the internalisation process were investigated and compared to the internalisation of this bacterium by osteoblasts.

6.2 Materials and Methods

6.2.1 Epithelial cell culture

The human cell line Hep2 was routinely cultured as described in chapter 2, section 2.1.

6.2.2 Bacterial strains and growth

Bacterial strains and growth conditions were described in chapter 2, section 2.3.

6.2.3 Internalisation assays and the role of *S. epidermidis* virulence factors in the internalisation process

To investigate the internalisation of *S. epidermidis* by epithelial cells and compare it to the internalisation of this bacterium by osteoblasts, the same experimental procedures described in section 3.2, chapter 3 were followed with the exception of using Hep2 cells instead of MG63 cells. The role of different *S. epidermidis* virulence factors in the internalisation of this bacterium by epithelial cells was also investigated as described for osteoblasts in chapter 4.

6.3 Results

6.3.1 Internalisation of different strains of *S. epidermidis* by epithelial cells

The uptake of *S. epidermidis* strain 19, NCTC11047, O-47and HB by cultured epithelial cells was examined. All strains of *S. epidermidis* tested in this experiment were internalised by epithelial cells (figure 6-1). The magnitude of internalisation of these strains of *S. epidermidis* by epithelial cells varied with the highest capacity of internalisation seen with strain 19. Internalisation of *S. epidermidis* strain 19 was 6-fold the internalisation of strain NCTC11047 and 4-fold that of strain O-47 (figure 6-1).

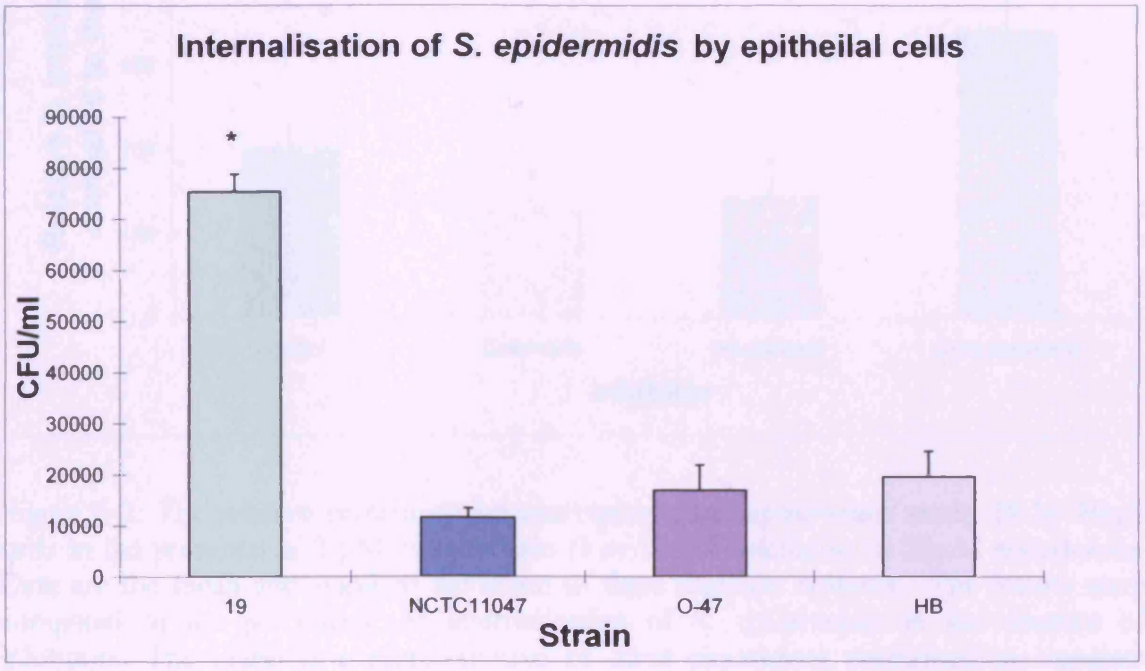

Figure 6-1: Colony-forming unit per millilitre of *S. epidermidis* strain 19, NCTC11047, O-47 and HB internalised by Hep2 cells at a multiplicity of infection of 200 to 1. Data are the mean and standard deviation of three replicate cultures. The graph is a representative of three experiments performed on separate occasions. * P value < 0.01.

6.3.2 The role of host cell microtubules and microfilaments in the internalisation of *S. epidermidis* by epithelial cells

To examine the role of host cell microfilaments and microtubules in the process of internalisation of *S. epidermidis* by cultured epithelial cells, colchicine, nocodazole and cytochalasin D were used. The inhibitors were added one hour before adding the bacteria.

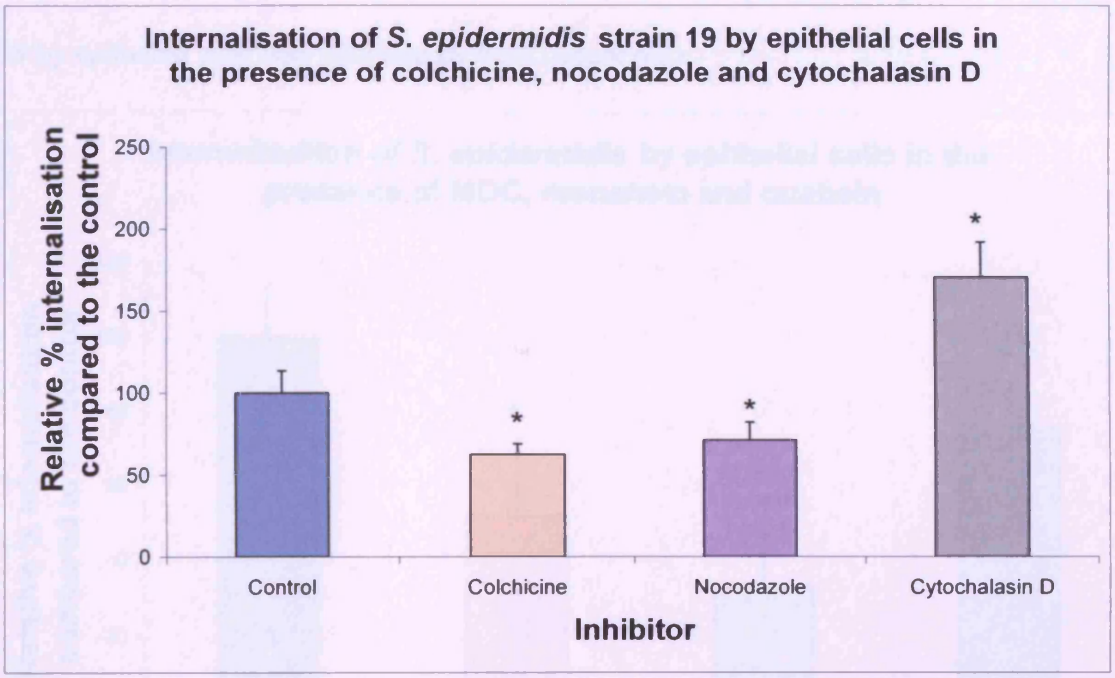

Figure 6-2: The relative percentage internalisation of S. *epidermidis* strain 19 by Hep2 cells in the presence of 2 μM cytochalasin D or 10μM colchicine or 20μM nocodazole. Data are the mean and standard deviation of three replicate cultures. The results were compared to the percentage of internalisation of *S. epidermidis* in the absence of inhibitors. The graph is a representative of three experiment performed on separate occasions. * P value < 0.05.

There was a significant 70% (P <0.01) increase in the internalisation of *S. epidermidis* strain 19 by epithelial cells in the presence of cytochalasin D which disrupts actin filaments in host cells. Depolymerisation of host cell microtubules using colchicine and

nocodazole significantly inhibited internalisation of *S. epidermidis* strain 19 by 37% and 28% respectively (figure 6-2).

6.3.3 The role of receptor recycling and endosome acidification in the internalisation of *S. epidermidis* by epithelial cells

To examine the effect of blocking host cell endosome acidification, internalisation assays were conducted in the presence of monensin. The uptake of *S. epidermidis* strain 19 by epithelial cells was inhibited by 66% (figure 6-3).

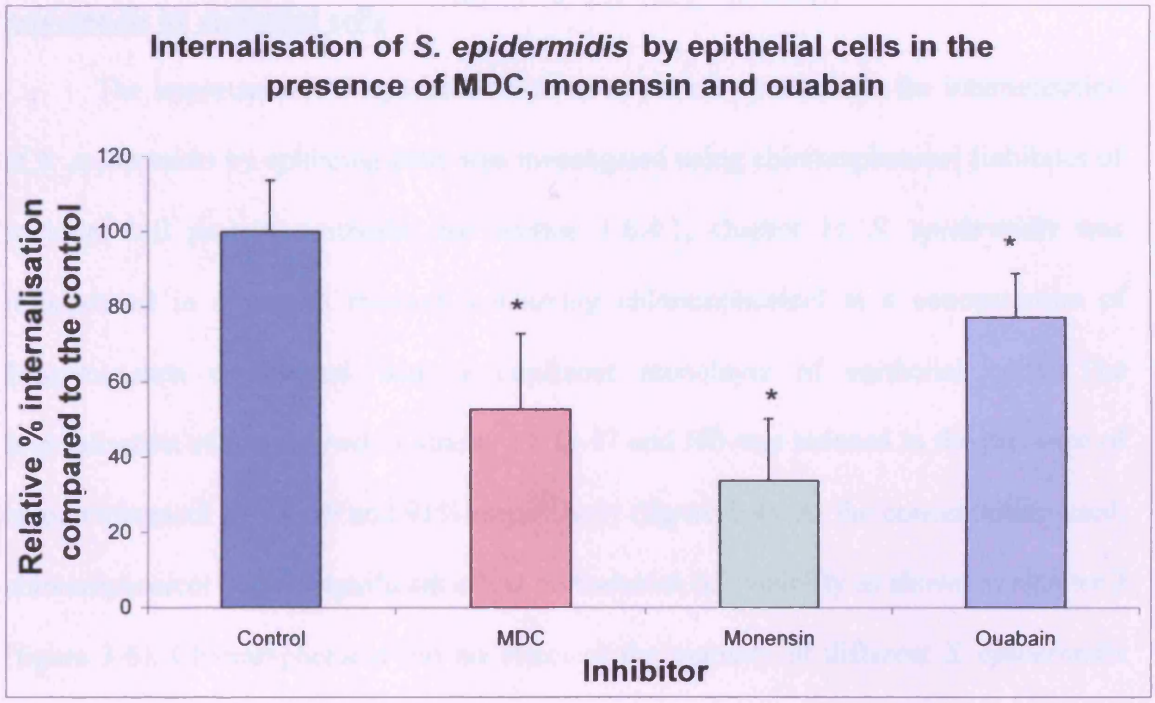

Figure 6-3: Internalisation of *S. epidermidis* strain 19 by Hep2 cells at an MOI of 200:1 in the presence of monensin or ouabain or monodansylcadaverine (MDC). Data are the mean and standard deviation of three replicate cultures. The graph is a representative of three experiments performed on separate occasions. * P value < 0.05.

The formation of coated pits can be inhibited by monodansylcadaverine (MDC) and ouabain (Dorn et al., 1998; Jevon et al., 1999; Biswas et al., 2000). Preincubation of

epithelial cells with MDC which inhibits transglutaminase activity and interferes with receptor recycling significantly reduced internalisation of *S. epidermidis* strain 19 by epithelial cells by 47% (figure 6-3). The addition of ouabain which blocks Na+/K+ ATPase causing arrest of coated pit formation by inhibiting the interaction of clathrin and adapter protein interactions reduced internalisation of *S. epidermidis* by epithelial cells by 20% (figure 6-3).

6.3.4 The role of de novo protein synthesis by bacteria in the internalisation of *S. epidermidis* by epithelial cells

The importance of *S. epidermidis* de novo protein synthesis in the internalisation of *S. epidermidis* by epithelial cells was investigated using chloramphenicol (inhibitor of bacterial cell protein synthesis, see section 1.6.4.1, chapter 1). *S. epidermidis* was resuspended in a growth medium containing chloramphenicol at a concentration of 10μg/ml then co-cultured with a confluent monolayer of epithelial cells. The internalisation of *S. epidermidis* strains 19, O-47 and HB was reduced in the presence of chloramphenicol by 93, 79 and 91% respectively (figure 6-4). At the concentration used, chloramphenicol had no significant effect on bacterial cell viability as shown in chapter 3 (figure 3-6). Chloramphenicol has no effect of the viability of different *S. epidermidis* strains as shown in chapter 3, figure 3-6.

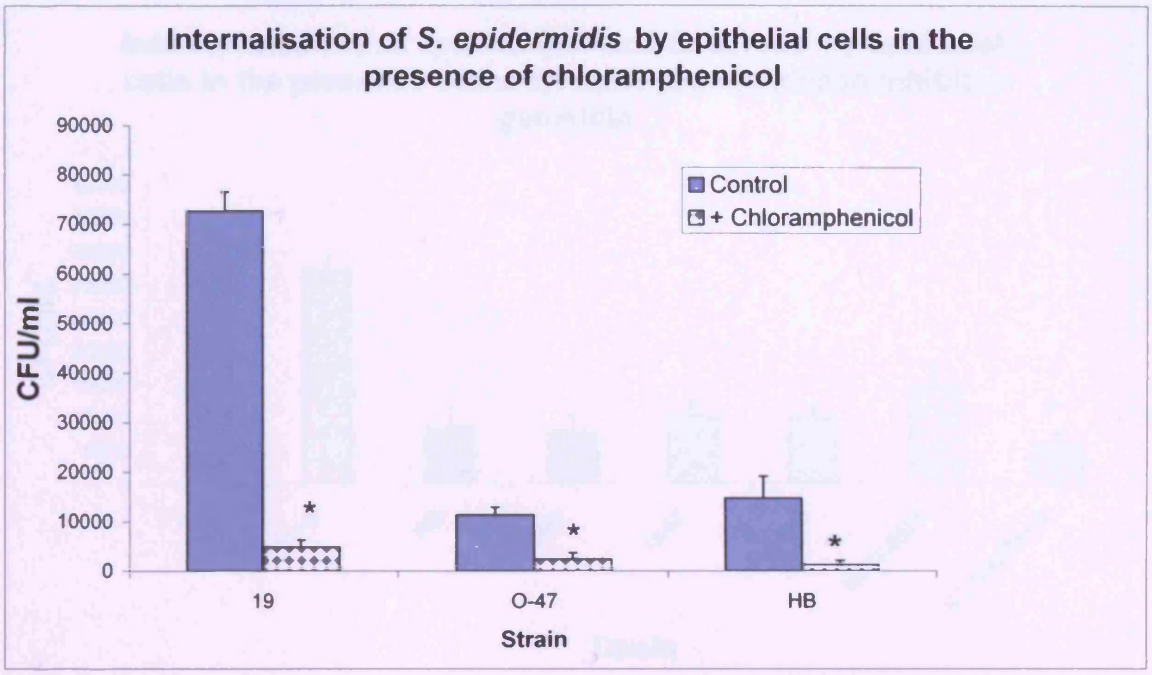

Figure 6-4: The effect of inhibiting *S. epidermidis* de novo protein synthesis on the internalisation of *S. epidermidis* strains 19, O-47 and HB by Hep2 cells. Data are the mean and standard deviation of three replicate cultures. The graph is a representative of three experiments performed on separate occasions. * P value < 0.05.

6.3.5 The role of tyrosine kinases in internalisation of *S. epidermidis* by epithelial cells

Genistein is a specific inhibitor of eukaryotic tyrosine protein kinases (TPK) and acts by inhibiting binding of ATP to the protein kinase (Almeida et al., 2000). Pretreatment of epithelial cells with genistein had no significant effect on the internalisation of the different *S. epidermidis* strains 19, HB or O-47 by epithelial cells (figure 6-5). In contrast internalisation of *S. aureus* by epithelial cells was significantly reduced by 60% (figure 6-5).

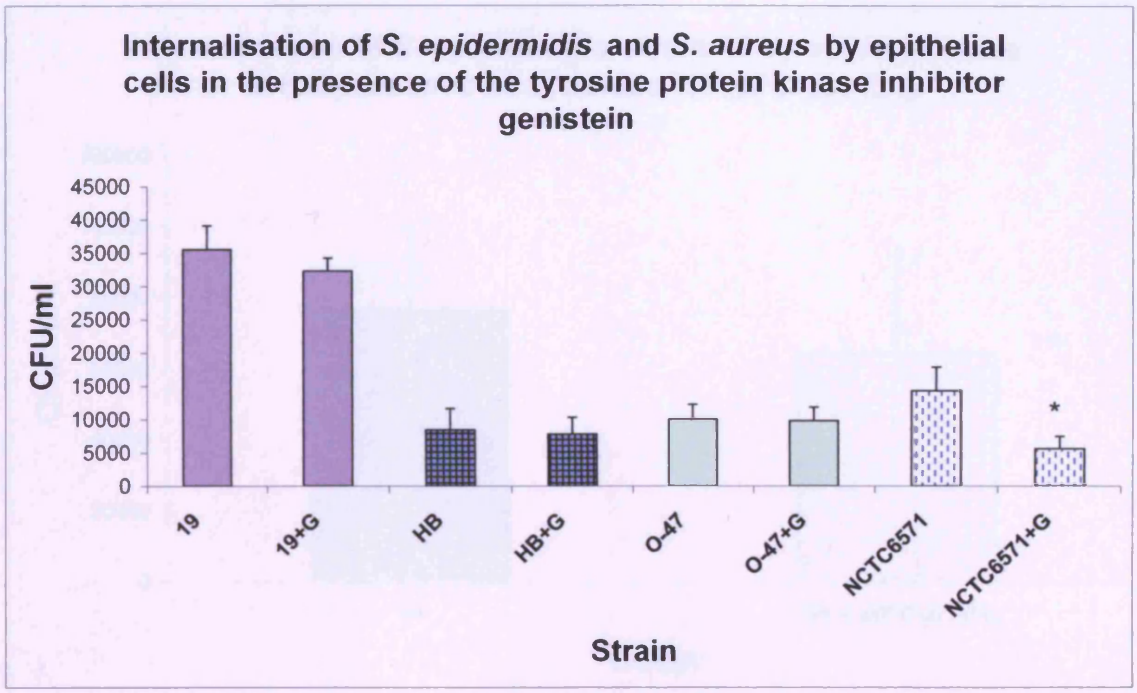

Figure 6-5: Colony-forming units per millilitre of different *S. epidermidis* strains and one *S. aureus* strain internalised by Hep2 cells in the presence of the tyrosine kinase inhibitor genistein (G). Data are the mean and standard deviation of three replicate cultures. The graph is a representative of three experiments performed on separate occasions. * P value < 0.05.

6.3.6 The effect of a fragment of the fibronectin binding protein from *S. aureus* on the internalisation of *S. epidermidis* by epithelial cells

Internalisation of *S. epidermidis* by epithelial cells was examined in the presence of a recombinant fragment of the *S. aureus* fibronectin binding protein rFnBPB[D1-D4] (figure 6-6). The *S. aureus* rFnBPB[D1-D4] did not inhibit the internalisation of *S. epidermidis* strain 19 by epithelial cells (figure 6-6)

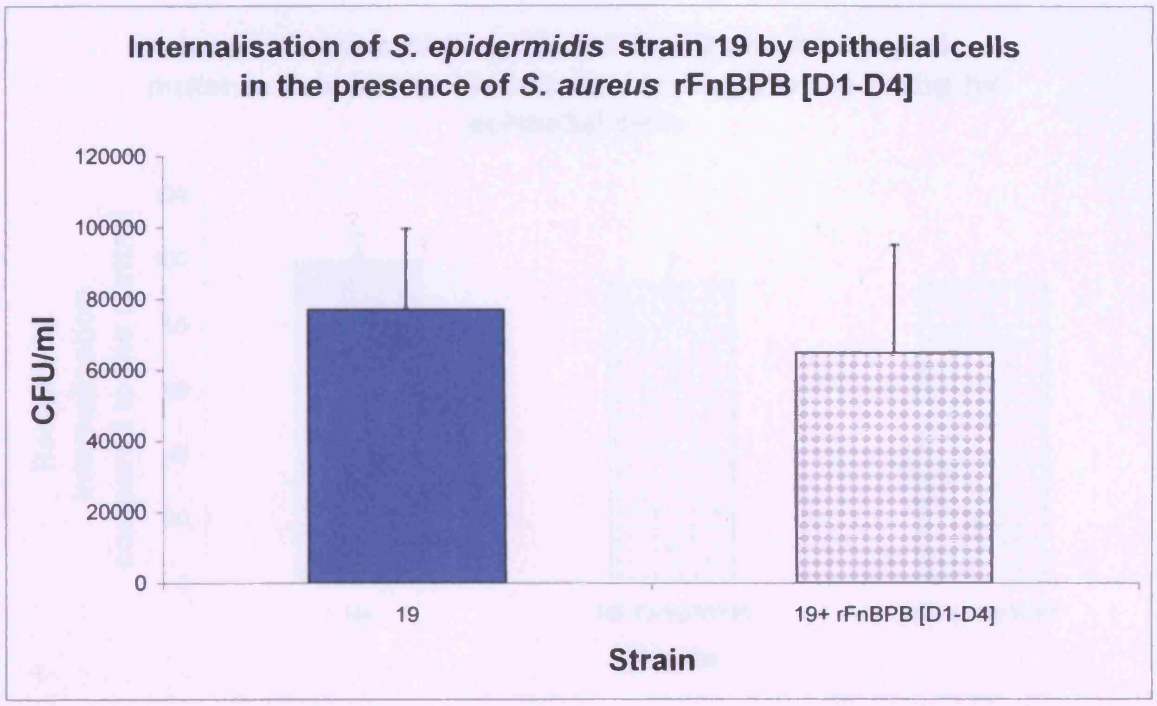

Figure 6-6: Internalisation of *S. epidermidis* strain 19 by epithelial cells in the presence of rFnBPB[D1-D4]. *S. epidermidis* was co-cultured with Hep2 cells at an MOI of 200:1. Data are the means and standard deviations of three replicate cultures. The graph is a representative of three experiments performed on separate occasions.

6.3.7 Internalisation of *S. epidermidis* strain HB and its isogenic mutants deficient in fibronectin binding protein by epithelial cells

S. epidermidis strain HB and its isogenic mutants deficient in the fibronectin binding protein Embp showed similar capacities to be internalised by epithelial cells (Figure 6-7). These findings suggest that the *S. epidermidis* Embp may not be involved in the internalisation of this bacterium by Hep2 cells.

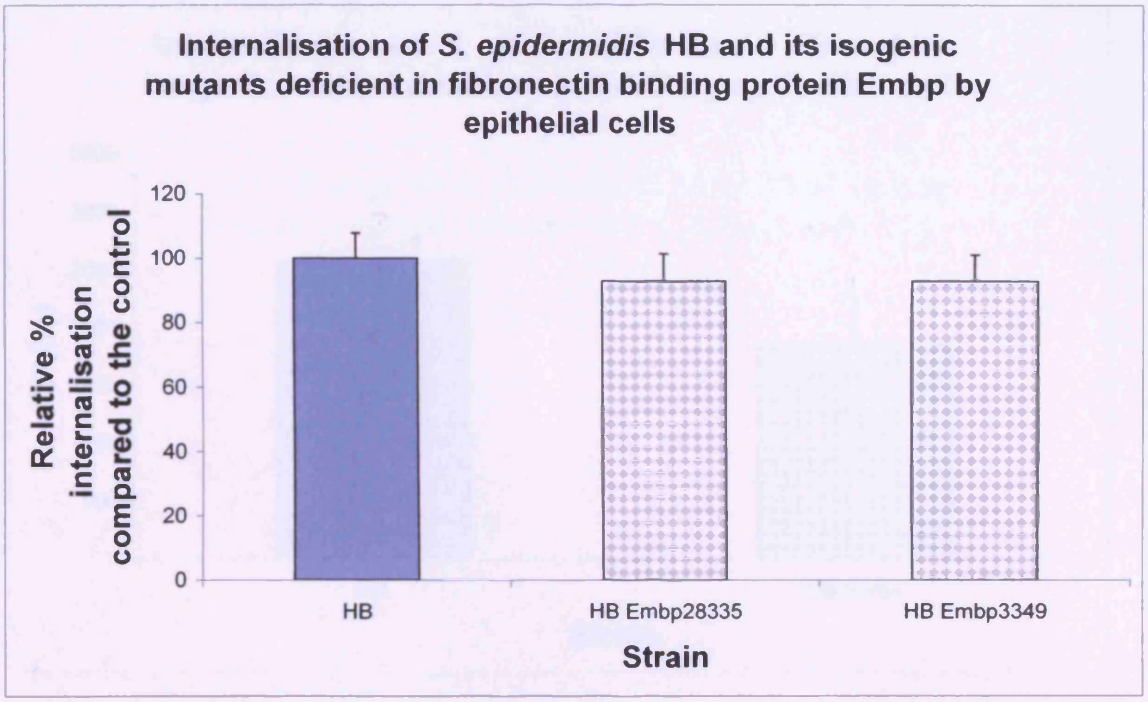

Figure 6-7: Internalisation of *S. epidermidis* strain HB and its isogenic mutants deficient in the fibronectin binding protein Embp by Hep2 cells. *S. epidermidis* strains were co-cultured with epithelial cells at an MOI of 200:1. Data are the means and standard deviations of three replicate cultures. The graph is a representative of three experiments performed on separate occasions.

6.3.8 The role of SdrG in the internalisation of *S. epidermidis* by epithelial cells

To determine if the MSCRAMM SdrG of *S. epidermidis* is involved in the internalisation of this organism by epithelial cells, *S. epidermidis* strain HB and its isogenic mutant deficient in SdrG were examined for their capacity to be internalised by epithelial cells. The SdrG deficient isogenic mutant of *S. epidermidis* showed a reduced level of internalisation compared to the parental wild type strain (figure 6-8).

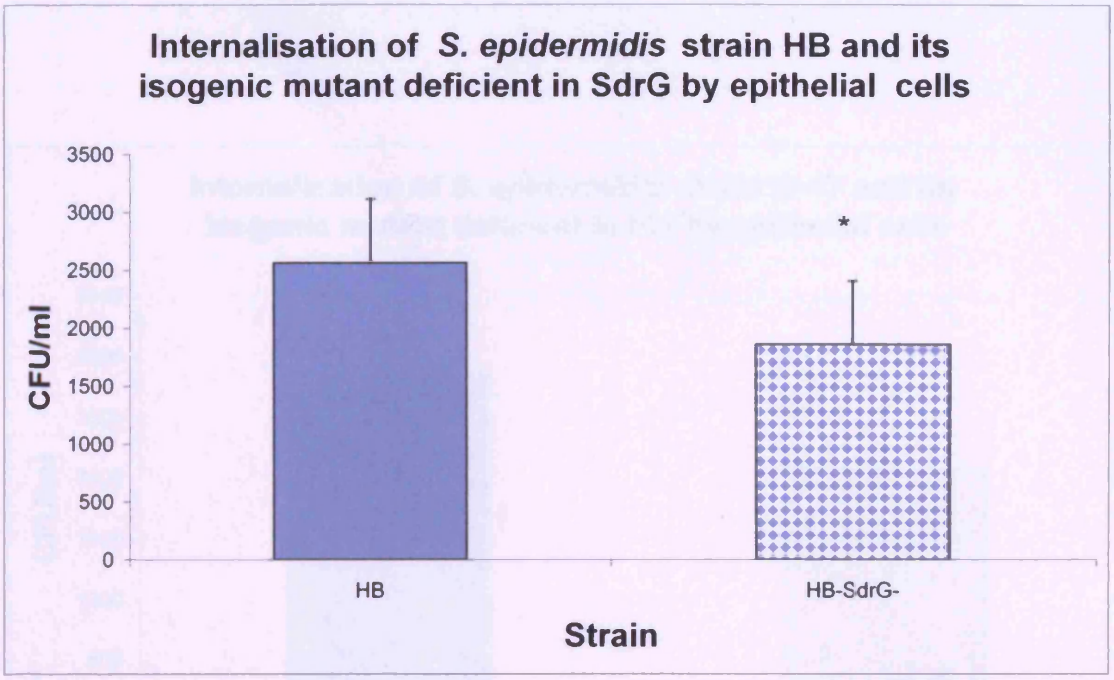

Figure 6-8: Internalisation of *S. epidermidis* strain HB and its isogenic mutant deficient in SdrG by epithelial cells. *S. epidermidis* strains were co-cultured with Hep2 cells at an MOI of 200:1. Data are the means and standard deviations of three replicate cultures. The graph is a representative of three experiments performed on separate occasions. * P value < 0.05.

6.3.9 PIA plays a role in the internalisation of *S. epidermidis* by epithelial cells

In this experiment we examined the role *of S. epidermidis* PIA in the internalisation of this bacterium by epithelial cells. An isogenic mutant of *S. epidermidis* O-47 with a Tn*917* insertion in the *ica* locus and which is therefore deficient in PIA production was used (Rupp et al., 2001). The *S. epidermidis* mutant deficient in PIA production showed a reduced capacity to be internalised by epithelial cells. There was a reduction of 27%, in comparison to the parental strain, in the capacity of the isogenic mutant to be internalised by epithelial cells (figure 6-9).

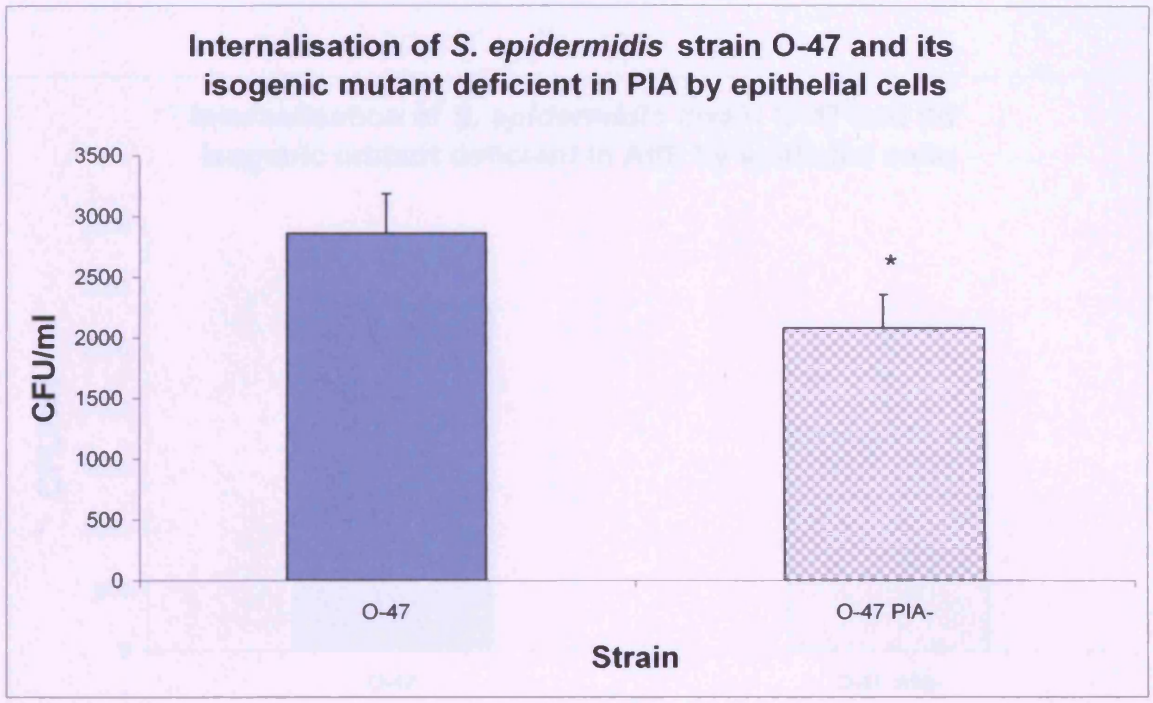

Figure 6-9: Internalisation of *S. epidermidis* strain O-47 and its isogenic mutant deficient in PIA by epithelial cells. *S. epidermidis* strains were co-cultured with Hep2 cells at an MOI of 200:1. Data are the means and standard deviations of three replicate cultures. The graph is a representative of three experiments performed on separate occasions. * P value < 0.05.

6.3.10 The autolysin AtlE plays a role in the internalisation of *S. epidermidis* by epithelial cells

The role of the *S. epidermidis* autolysin AtlE in internalisation of this bacterium by epithelial cells was investigated in this study. An isogenic mutant of *S. epidermidis* strain O-47 with a Tn*917* insertion in the *AtlE* gene and which therefore was deficient of AtlE was used in this experiment. The level of internalisation of the isogenic mutant was

compared to that of wild type parental strain (figure 6-10). Internalisation of the AtlE mutant by epithelial cells was reduced by 36% compared to the wild type strain

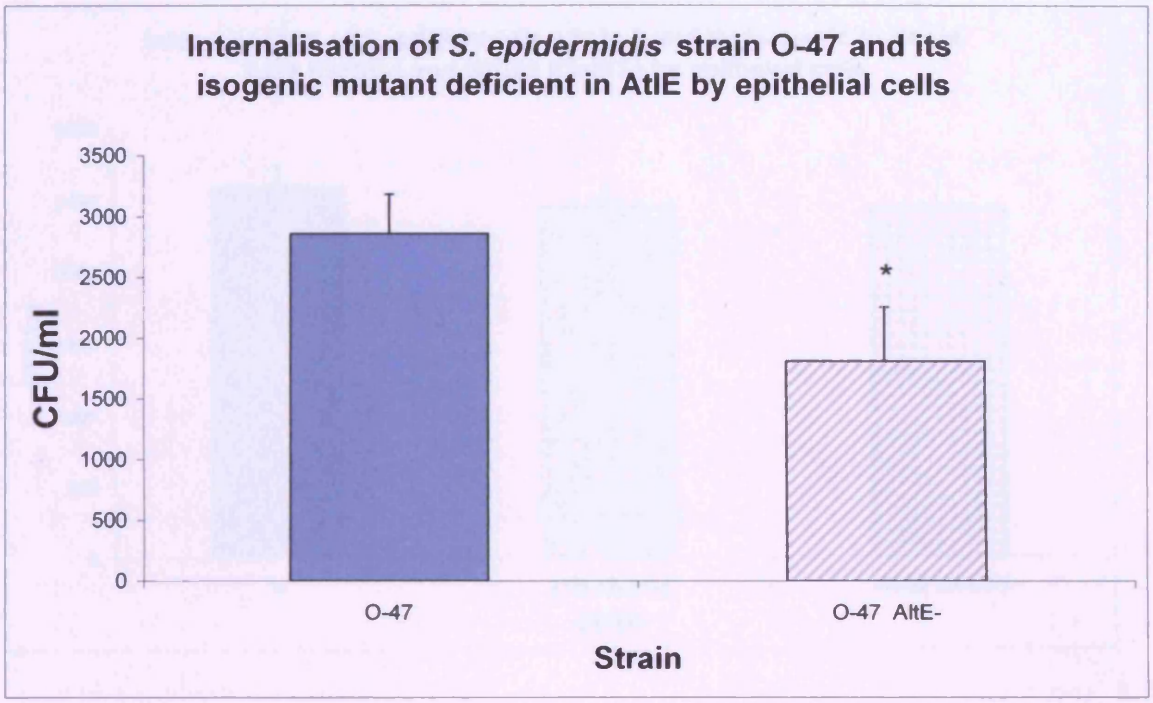

Figure 6-10: Internalisation of *S. epidermidis* strain O-47 and its isogenic mutant deficient in AtlE by Hep2 cells. *S. epidermidis* strains were co-cultured with epithelial cells at an MOI of 200:1. Data are the means and standard deviations of three replicate cultures. The graph is a representative of three experiments performed on separate occasions. * P value < 0.05.

6.3.11 The GehC and GehD lipases are not involved in the internalisation of *S. epidermidis* by epithelial cells

It has been postulated that the lipases of *S. epidermidis* are virulence factors of this bacterium. To examine the role of the two known lipases produced by *S. epidermidis* in the internalisation of this bacterium by epithelial cells, *S. epidermidis* isogenic mutants deficient in either GehD (KIC82) or GehC (2J24) were compared to the wild type

parental strain in internalisation assays. Figure 6-11 shows that neither of the lipases contributed significantly to the internalisation of *S. epidermidis* by epithelial cells.

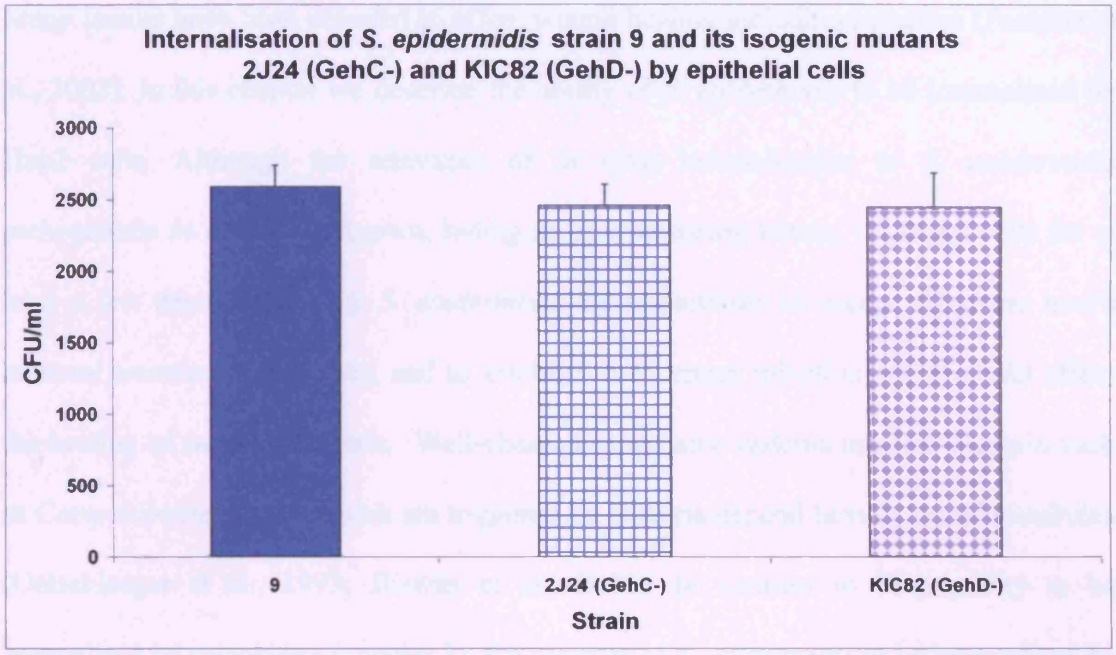

Figure 6-11: Internalisation of *S. epidermidis* strains 9, 2J24 (GehC-), and KIC82 (GehD-) by epithelial cells. *S. epidermidis* strains were co-cultured with Hep2 cells at an MOI of 200:1. Data are the means and standard deviations of three replicate cultures. The graph is a representative of three experiments performed on separate occasions.

6.4 Discussion

Surgical wound healing is a dynamic process in which epithelial cells play a major role. The integrity of the surgical wound is very critical for successful surgery. Many factors have been reported to affect wound healing including infection (Pessaux et al., 2003). In this chapter we describe the ability of *S. epidermidis* to be internalised by Hep2 cells. Although the relevance of *in vitro* internalisation to *S. epidermidis* pathogenesis *in vivo* is not known, hiding in, and persisting within, epithelial cells for at least a few days would give *S. epidermidis* the opportunity to escape from the host's humoral immune mechanisms and to establish a recurrent infection which could affect the healing of surgical wounds. Well-characterised entry systems in some bacteria such as *Campylobacter jejuni*, which are triggered by bacteria depend heavily on microtubules (Oelschlaeger et al., 1993; Biswas et al., 2000). In contrast to its capacity to be internalised by osteoblasts (chapter 3), the capacity of *S. epidermidis* to be internalised by epithelial cells was reduced in the presence of microtubule inhibitors such as colchicine and nocodazole (figure 6-2). Cytochalasin-induced actin disruption has been typically associated with decreased bacterial internalisation by cultured epithelial cells (Lamaze et al., 1997; Ellington et al., 1999; Jevon et al., 1999; Marouni and Sela, 2004). It has been reported that the number of some intracellular bacteria such as *Salmonella typhimurium* and *Escherichia coli* can be increased in the presence of cytochalasin D (Wells et al., 1998). It was reported in chapter 3 that the capacity of some strains of *S. epidermidis* such as strains 19 and NCTC11047 to be internalised by osteoblasts was increased in the presence of cytochalasin D. Some possible reasons for this increase in the number of intracellular *S. epidermidis* were also discussed in section 3.4. in the experiments presented in this chapter we show that cytochalasin D increased the numbers of *S.*

epidermidis strain 19 in epithelial cells (figure 6-2). The increase in numbers of *S. epidermidis* within epithelial cells in the presence of cytochalasin D were less than those observed with osteoblasts (chapter3). The reason for the difference between epithelial cells and osteoblasts is not clear but could be due to a difference in the response of these cell types to cytochalasin D and/or difference in the mechanism of uptake of *S. epidermidis* by different host cells.

The inhibition of endosome acidification by monensin reduced the ability of *S. epidermidis* to be internalised by epithelial cells. This is very unlikely to be due to a direct bactericidal effect of monensin, since monensin has been shown not to affect the viability of bacteria (Oelschlaeger and Tall, 1996). However, the inhibitory effect of monensin on the internalisation of *S. epidermidis* by epithelial cells was greater than that observed for the internalisation of this bacterium by osteoblasts (chapter 3). The trapping of receptors used by host cells to trigger internalisation in unacidified endosomes (acidification releases endosome-bound receptors and allows for recycling to the cell surface) may be the cause of the monensin effect.

Reduced uptake into monodansylcadaverine treated epithelial cells might additionally or solely be due to the inhibitory effect of MD on transglutaminases essential for receptor recycling, leading to depletion of surface-exposed receptors necessary for efficient uptake of *S. epidermidis.* Nevertheless, reduced internalisation of *S. epidermidis* into MD-treated epithelial cells suggests that receptors recycled through endosomes are involved in the uptake of this bacterium into epithelial cells. Taken together, these results suggest that *S. epidermidis* triggers an uptake mechanism(s) into epithelial cells that involves host cell

microtubules and a receptor(s) which is recycled through endosomes and reflects receptor-mediated endocytosis. De novo protein synthesis is necessary for efficient invasion by *S. epidermidis*, as seen for a variety of bacteria, and may reflect the synthesis of bacterial ligands with short halflives necessary for uptake. The tyrosine protein kinases were not involved in the internalisation of *S. epidermidis* by epithelial cell. A similar find was also made in the case of the internalisation of *S. epidermidis* by osteoblasts (chapter4).

In chapter 4 the role of different *S. epidermidis* virulence factors in the process of internalisation of this bacterium by osteoblasts was examined. The same factors were examined in this chapter for their possible role in the internalisation of *S. epidermidis* by epithelial cells. Similar findings were found with epithelial cells. The *S. epidermidis* SdrG, PIA and autolysin AtlE were involved in the process of internalisation of this bacterium by epithelial cells. The fibronectin binding protein Embp, GehC and GehD lipases of *S. epidermidis* were not involved in the internalisation of this bacterium by epithelial cells. A brief comparison between the internalisation of *S. epidermidis* by osteoblasts and epithelial cells is shown in table 6-1.

Table 6-1: A summary of the effect of disruption of host cell or bacterial function

Structure/product/process	**Osteoblast**	**Epithelial cell**
Microtubule polymerisation	-	+
Microfilament polymerisation	*	*
Receptor recycling	+	+
De novo protein synthesis	+	+
Tyrosine protein kinases	-	-
S. aureus rFnBPB[D1-D4]	-	-
Embp	-	-
SdrG	+	+
PIA	+	+
The autolysin AtlE	+	+
GehC lipase	-	-
GehD lipase	-	-

(+) Played a role in the internalisation process	(-) Has no role in the internalisation process	(*) Strain dependent effect [either (+) or (-)]

6.5 Conclusions

In chapter 3 it was reported that bone cells internalise *S. epidermidis*. In this chapter it was shown that epithelial cells are also capable of internalising *S. epidermidis* using uptake mechanisms that are distinct from those employed in the uptake of *S. aureus*. The uptake of *S. epidermidis* in bone and epithelial cells can differ depending on the strains of this bacterium. Although host cell microtubules played a role in the internalisation of *S. epidermidis* by epithelial cells, no significant role was seen for the uptake of this bacterium by osteoblasts. Virulence determinants such as SdrG, PIA and AtlE were involved in the process of uptake of *S. epidermidis* by both osteoblasts and epithelial cells. The uptake of *S. epidermidis* by epithelial cells may be a consideration when dealing with surgical wound-related infections.

Chapter 7

Chapter 7

Adhesion of *S. epidermidis* to surgical sutures

7.1 Introduction

Advances in surgical procedures and aseptic techniques have resulted in a reduction in the incidence of surgical related infections, but such infections do still occur at unacceptably high incidence and are complicated in the presence of implanted devices (Rothenburger et al., 2002). The situation becomes more difficult when applied to intra-oral wounds where asepsis is a challenge. It has been accepted that bacterial adhesion to surfaces is a prerequisite step in foreign body infections (Gristina and Costerton, 1985). Sutures are considered the most common implantable devices due to their diverse usage in surgical procedure (Gabrielli et al., 2001) and can be classified into two types according to their ability to be absorbed by host tissue, absorbable and non-absorbable. They are used either in elective surgery where asepsis is performed prior to any incision, or in contaminated wounds where asepsis control is difficult. Non-absorbable sutures are usually removed after seven days in order to allow wound healing. The absorption time for some sutures such as polydioxanone can be as long as 180 days (Molea et al., 2000). Although sutures are considered to be an important predisposing factor in the development of infections, few studies have been conducted in this area of research (Howell et al., 1995; Gabrielli et al., 2001; Otten et al., 2005). Keratitis following eye surgery can be caused by bacterial infiltration due to irritation of loose, exposed or broken sutures. Such infections may have serious complications including visual loss (Christo et al., 2001). Comparisons between infections of wounds closed by either sutures, or tissue adhesives have shown that there are more staphylococcal bacterial counts where sutures have been used (Howell et al., 1995).

Although the mechanism behind the increased risk of wound sepsis in the presence of suture materials is not clear, it is generally thought that sutures act as adhesive surfaces that promote bacterial accumulation. Adherence of *S. epidermidis* and *S. aureus* to cardiac sutures has been suggested to be one of the explanations for these organisms being the commonest cause of early prosthetic valve endocarditis (Shuhaiber et al., 1989). Bacterial cell surface properties can affect adhesion to different biomaterials (Vacheethasanee et al., 1998). Suture use not only plays a role in infection but triggers inflammatory reactions, producing scaring and altering the process of wound healing (Gabrielli et al., 2001).

Two stages have been identified in the process of foreign body related infection and biofilm formation. These are the primary attachment of bacteria to the material and the formation of multi-layered cell clusters. Very few *S. epidermidis* virulence factors have been identified. However the role of a few of the known virulence factors in attachment to biomaterials has been reported in the literature. *S. epidermidis* MSCRAMMS, such as the fibrinogen binding protein SdrG (Hartford et al., 2001), and the fibronectin binding protein Embp (Williams et al., 2002), might be involved in indirect attachment of *S. epidermidis* to biomaterials. The *S. epidermidis* autolysin AtlE has been found to be responsible for the primary attachment of this organism to central catheters (Rupp et al., 2001). The polysaccharide intercellular adhesin (PIA) of *S. epidermidis* has been demonstrated to be involved in intercellular adhesion, biofilm formation and biomaterial related infection (Rupp et al., 1999). The GehD lipase of *S. epidermidis* has been shown to be a bifunctional molecule, acting both as a lipase and a collagen adhesin (Bowden et

al., 2002). However, the role of this adhesin in adhesion to abiotic surfaces has not been investigated.

In this study, we have examined the capacity of different strains of *S. epidermidis* to adhere to commonly used surgical sutures. The role of some *S. epidermidis* virulence factors in the capacity of this organism to adhere to surfaces was also determined. These observations could yield insights into mechanisms relevant to suture related infection and help in the prevention of such infections.

7.2 Materials and Methods

7.2.1 Bacterial strains and growth

Bacterial strains and the growth conditions used in this study were described in chapter 2, section 2.3.1.1.

7.2.2 Suture materials

Five sutures were used in this study; these were prolene, vicryl, silk (mersilk), monocryl and polydioxanone (PDS II), all manufactured by Ethicon and obtained from Johnson and Johnson Company, UK. Prolene (Polyproplene) is a synthetic monofilament which is nonabsorbable. Vicryl (Polyglactin 910) is a synthetic, absorbable suture, composed of 90 % glycolide and 10 % L-lactide. Mersilk is a nonabsorbable multifilament, composed of an organic protein derived from *Bombyx mori* of the family Bombycidae. Monocryl (Poliglecaprone 25) is a synthetic, absorbable, monofilament

suture prepared from a copolymer of glycolide and caprolactone. PDS II (Polydioxanone) is a synthetic, absorbable, monofilament suture made of the polyester poly p-dioxanone.

7.2.3 Adhesion assay

Sterile suture samples of two centimeter length were incubated with *S. epidermidis* strains in 4 milliliters of sterile phosphate buffered saline (PBS) (Sigma-Aldrich, UK). Incubation was at 37°C with shaking at 200 rpm for 3 hours unless otherwise specified. After incubation, non adhered bacteria were removed by washing with PBS three times. As a control, the liquid from the last wash step was examined for bacterial colonies by plating on blood agar plates containing 5% horse blood. To remove the adherent bacteria, the samples were resuspended into two milliliters of sterile PBS containing 0.1% Triton X100 and vortexed for three minutes. The number of bacteria adhered to sutures was determined by serial dilution and plating on blood agar plates containing 5% horse blood. After removal of adhered bacteria the sutures were gently rolled on blood agar plates to enumerate any remaining adhered bacteria.

7.2.4 Adhesion of *S. epidermidis* to plasma coated sutures

Two centimetre pieces of surgical sutures were incubated in human plasma (Sigma, UK) in 5 ml tubes overnight at 4°C. Sutures were washed three times with PBS then resuspended into 5 ml of sterile PBS and 10^8 bacteria were added. Tubes were incubated at 37°C with shaking for 3 hours. Sutures were washed 3 times with PBS to remove unattached bacteria. The last wash was plated as serial dilutions on blood agar to confirm the removal of non adherent bacteria. Sutures were resuspended into 3 ml of fresh PBS containing 0.1 % Triton X-100 then vortexed for 3 minutes. Bacterial numbers

adhered to sutures were determined by plating serial dilutions on agar plates containing 5% horse blood.

7.2.5 Scanning electron microscopy (SEM)

Adhesion of *S. epidermidis* to different surgical sutures was examined using an electron microscope. Specimens were fixed with 4% paraformaldehyde and mounted flat for surface scanning. They were examined using a Cambridge stereoscan 90B scanning electron microscope (Polaron, Cambridge, UK). Micrographs were taken at 400x and 2000x magnification.

7.3 Statistics

All data are shown as the mean ± the standard deviation. Data were compared using Student's t-test when normally distributed or Mann Whitney U test when data were skewed. A P value of less than 0.05 was considered statistically significant.

7.4 Results

7.4.1 Different strains of *S. epidermidis* have different capacities to adhere to suture materials

To determine if *S. epidermidis* adheres to surgical suture materials, five different sutures were incubated with different strains of *S. epidermidis*. Figure 7-1 shows the capacity of three *S. epidermidis* strains to adhere to five suture materials, prolene, mersilk, monocryl, polydioxanone (PDS II) and vicryl. *S .epidermidis* strain NCTC11047 had a significantly greater capacity to bind to of all the sutures tested when compared to strains 19 and RP62A (figure 7-1). Adhesion of *S. epidermidis* NCTC11047 to monocryl suture was 8-fold higher than strain 19 ($P< 0.01$). Adhesion of *S. epidermidis* to sutures differed according to the material from which the suture was constructed. For example adhesion of *S. epidermidis* strain NCTC11047 to silk was 5.4 fold higher than to polydioxanone ($P< 0.01$). *S. epidermidis* strains NCTC11047, 19 and RP62A showed significantly higher levels of adhesion to both vicryl and silk when compared to their binding to other sutures.

Adhesion of *S. epidermidis* strain 19 to silk was 12-fold higher than its adhesion to polydioxanone ($P< 0.001$). Comparison of the adhesion of *S. epidermidis* strain 19 to all five sutures revealed that adhesion to PDS II was lowest followed by prolene, monocryl, vicryl and silk. *S. epidermidis* strains NCTC11047 and RP62A showed low adhesion to PDS II and prolene. These findings suggest that the monofilament sutures PDS II, monocryl and prolene to which less bacteria bind may present less of an infection risk than multifilament sutures such as vicryl and silk.

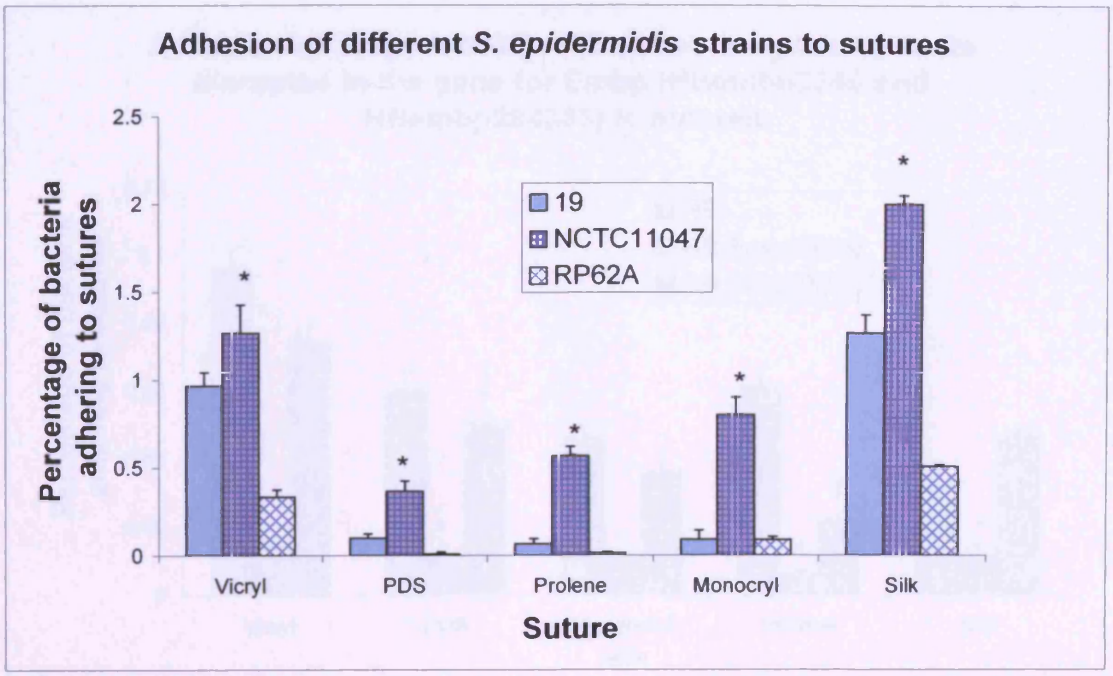

Figure 7-1: Adhesion of *S. epidermidis* strains 19, NCTC11047 and RP62A to sutures. Data are the percentage of the bacterial inoculums adhering to sutures. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate cultures. * P value < 0.05.

7.4.2 *S. epidermidis* fibronectin binding protein Embp affects bacterial adhesion to suture materials

A *S. epidermidis* isogenic mutant with a disruption at the start of the gene for Embp (HB-Embp3349) had a significantly decreased capacity to bind to sutures compared to the wild type parental strain HB or an isogenic mutant with a disruption in the 3' prime end of the gene coding for Embp (HB-Embp284335) (figure 7-2)

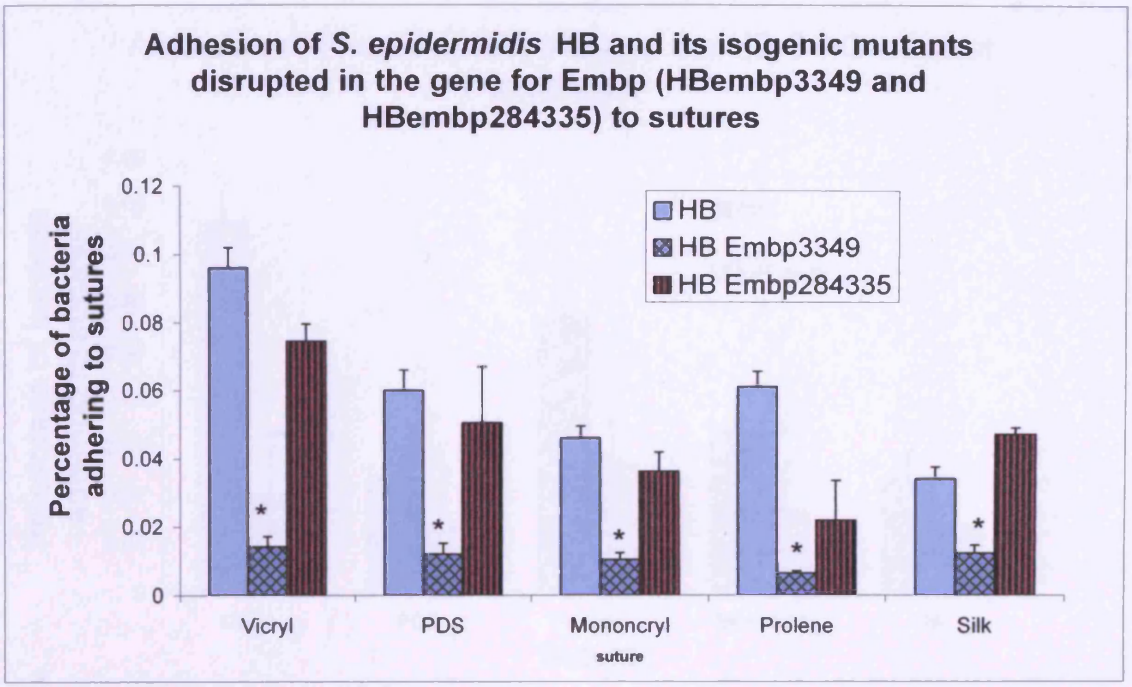

Figure 7-2 : Adhesion of *S. epidermidis* strain HB and its isogenic mutants disrupted in the gene for the fibronectin binding protein Embp to sutures. Data are the percentage of the bacterial inoculums adhering to sutures. The results are from one representative experiment of at least three; data are the means and standard deviations of three replicate experiments. * P value < 0.05.

7.4.3 *S. epidermidis* SdrG is involved in adhesion to suture materials

It has been reported that *S. epidermidis* SdrG (fbe) promotes bacterial adhesion to fibrinogen (Hartford et al., 2001). To determine if this virulence factor was involved in adhesion of *S. epidermidis* to suture materials, a *S. epidermidis* mutant disrupted in the *sdrG* gene was compared to the parental strain (HB). Figure 7-3 shows the levels of adhesion of *S. epidermidis* strain HB and its SdrG deficient mutant to five different surgical sutures. There was a significant reduction in the adhesion of the mutant strain to all of the sutures. The level of adhesion was reduced by 28 to 70 % dependent on the suture material. Adhesion of the parent strain to vicryl was 70% higher than its isogenic mutant (P = 0.0001).

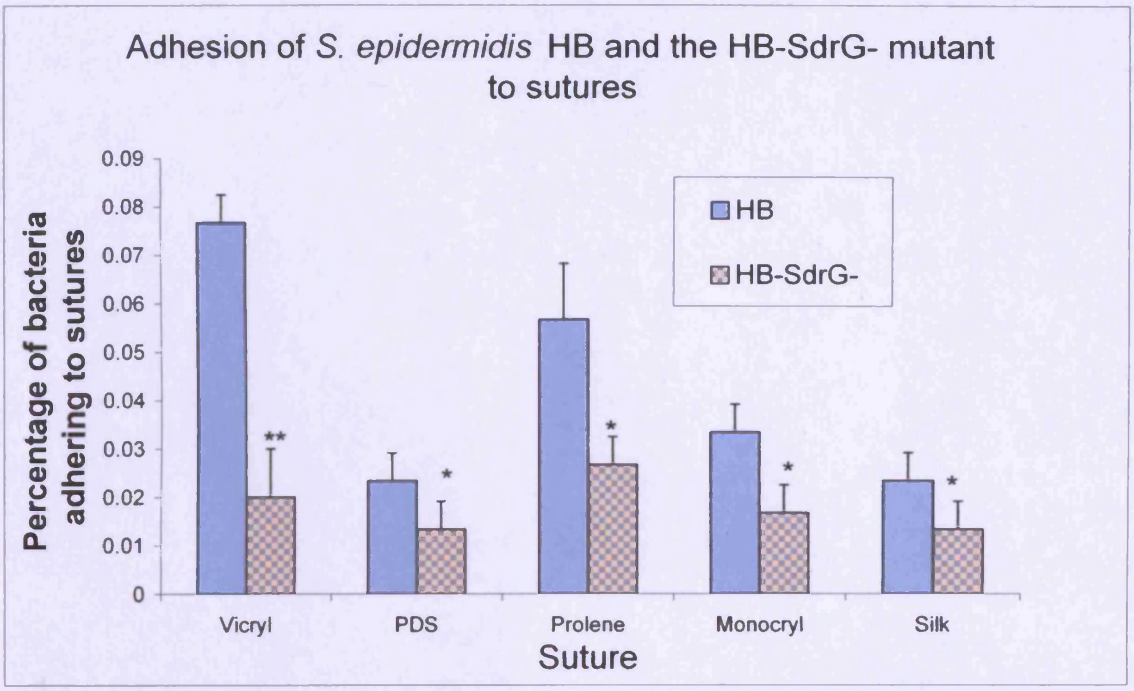

Figure 7-3: The graph shows the capacity of *S. epidermidis* HB and its isogenic mutant disrupted in SdrG to adhere to sutures. The experiment is a representative of three experiments performed on different occasions. Data are the means and standard deviations of three replicate experiments.* indicates P value < 0.05, ** P value < 0.01.

7.4.4 The GehD lipase is involved in adhesion of *S. epidermidis* to suture materials

The genes for two lipases GehC and GehD from *S. epidermidis* have been cloned and sequenced (Simons et al., 1998; Longshaw et al., 2000). The lipase GehD has been found to be a bifunctional molecule, not only acting as a lipase but also as a cell surface-associated collagen adhesin (Bowden et al., 2002). We examined the role of the *S. epidermidis* lipases in adhesion of this bacterium to different suture materials. Figure 7-4 shows the adhesion of *S. epidermidis* strain 9, and its isogenic mutants 2J24, deficient in GehC lipase, and KIC82 deficient in GehD to sutures. The GehD mutant was significantly less adherent to sutures when compared to the wild type strain. The reduction of adhesion ranged from 68% for vicryl ($P < 0.01$) to 88% for PDS ($P = 0.001$).

There was no significant reduction in the adhesion of the GehC mutant to sutures compared to the parental strain (figure 7-4).

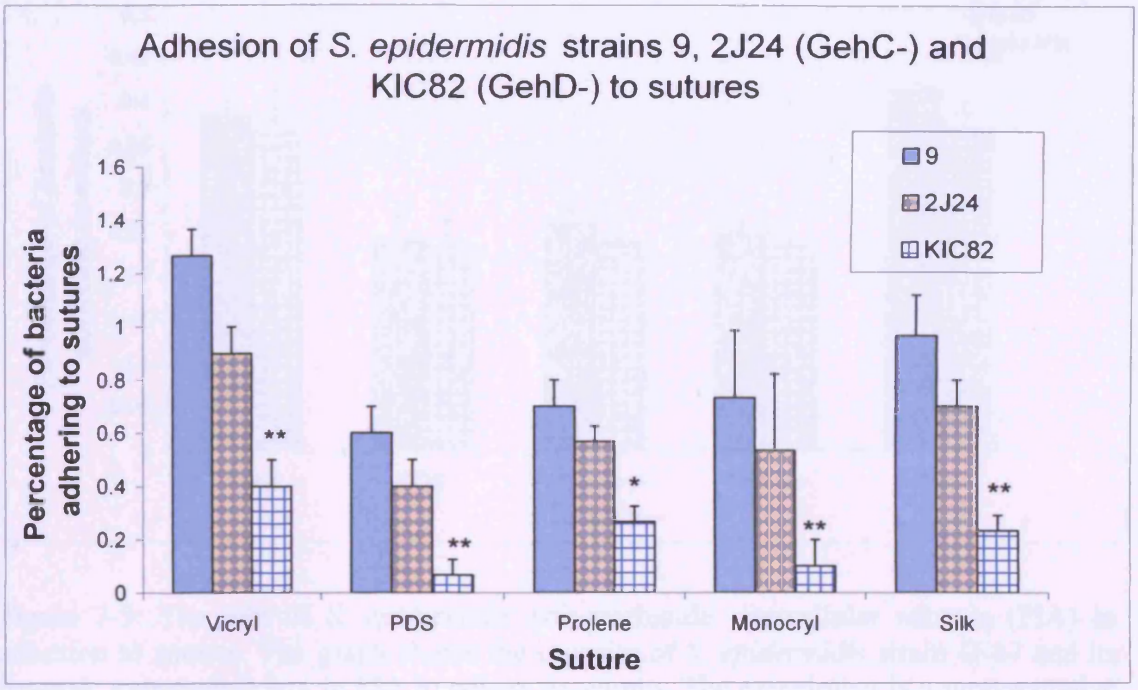

Figure 7-4: The role of *S. epidermidis* lipases in adhesion to sutures. The graph shows the capacity of *S. epidermidis* strain 9, its isogenic mutants 2J24 deficient in the GehC lipase and KIC82 deficient in the GehD lipase to adhere to sutures. The experiment is a representative of three experiments performed on different occasions. Data are the means and standard deviations of three replicate experiments. * indicates p value < 0.05, ** P value < 0.01.

7.4.5 *S. epidermidis* PIA is not involved in adhesion to suture materials

Polysaccharide intercellular adhesin (PIA) has been shown to be important in biofilm formation and catheter related infection (Rupp et al., 2001). In this experiment we investigated the role of PIA in adhesion of *S. epidermidis* to sutures. Figure 7-5 shows that disruption of PIA production in *S. epidermidis* did not have a significant effect on the adhesion of bacteria to sutures.

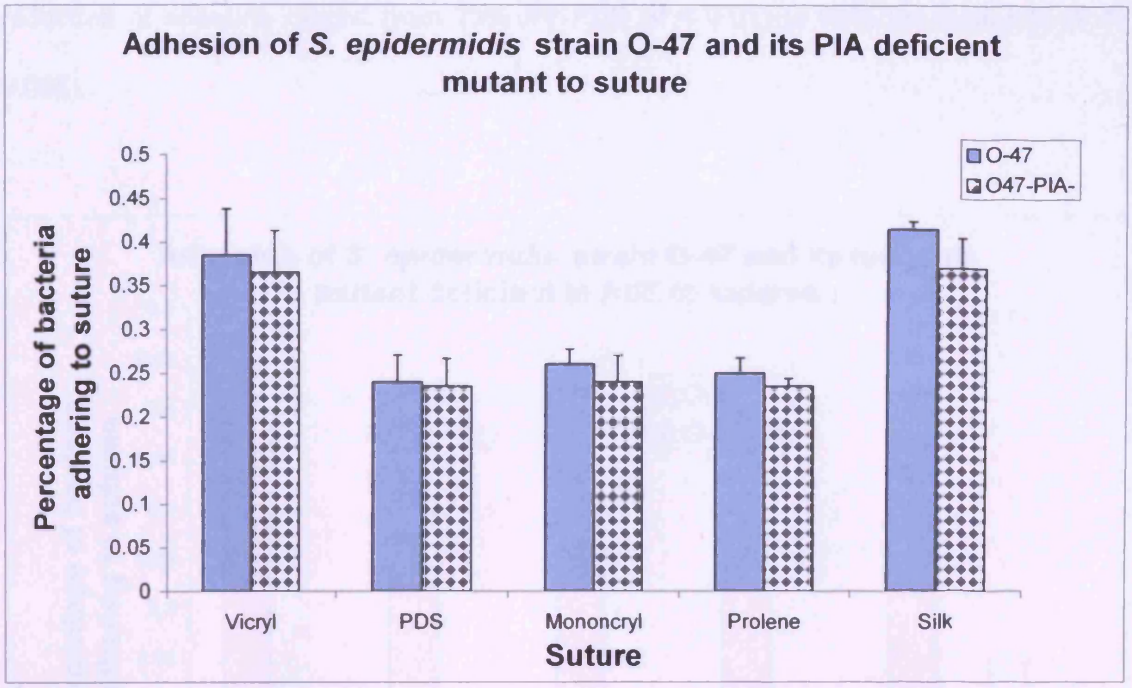

Figure 7-5: The role of *S. epidermidis* polysaccharide intercellular adhesin (PIA) in adhesion to sutures. The graph shows the capacity of *S. epidermidis* strain O-47 and its isogenic mutant deficient in PIA to adhere to sutures. The experiment is a representative of three experiments performed on different occasions. Data are the means and standard deviations of three replicate experiments.

7.4.6 The *S. epidermidis* autolysin AtlE plays a role in bacterial adhesion to suture materials

The *S. epidermidis* autolysin AtlE can mediate primary attachment of *S. epidermidis* to polymer surfaces (Rupp et al., 2001). In this experiment we examined the role of the autolysin AtlE in the adhesion of *S. epidermidis* to different surgical sutures. Figure 7-6 shows adhesion of *S. epidermidis* strain O-47 and an isogenic mutant deficient in the autolysin AtlE to five different sutures. Disruption of the gene for the autolysin AtlE significantly reduced adhesion of *S. epidermidis* to all of the sutures tested. The

reduction of adhesion ranged from 75% for PDS ($P < 0.01$) to 95% for monocryl ($P = 0.008$).

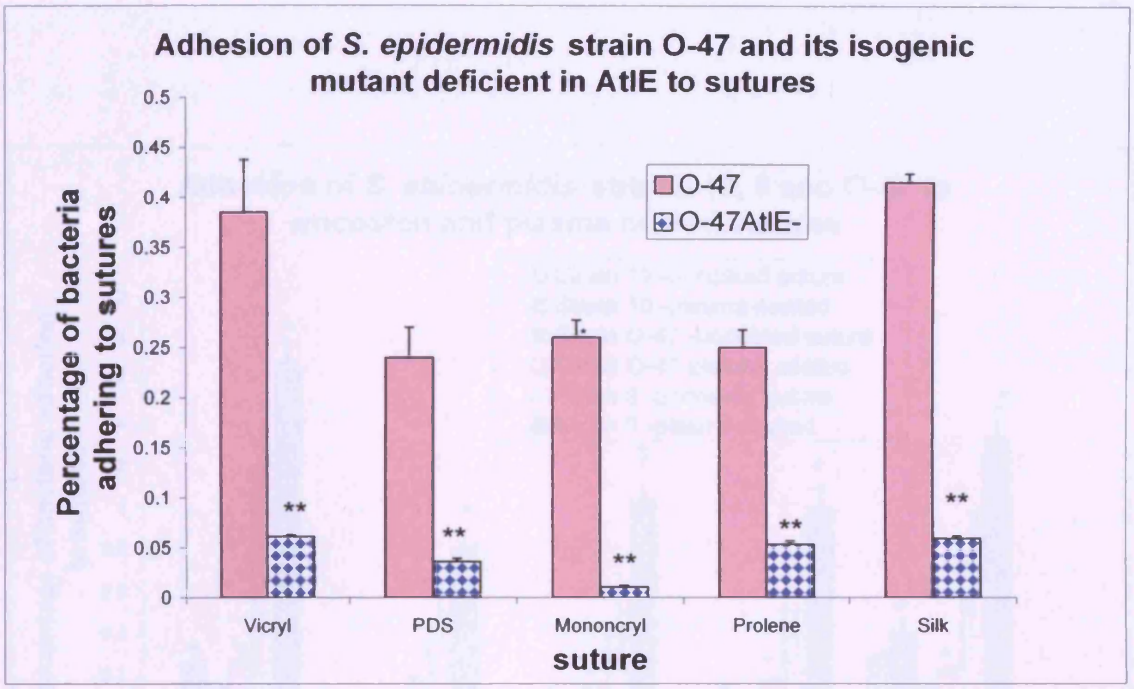

Figure 7-6: The role of the *S. epidermidis* autolysin AtlE in adhesion to sutures. The graph shows the capacity of *S. epidermidis* strain O-47 and its isogenic mutant deficient in the autolysin AtlE to adhere to sutures. The experiment is a representative of three experiments performed on different occasions. Data are the means and standard deviations of three replicate experiments. ** indicates P value < 0.01.

7.4.7 *S. epidermidis* adheres to plasma coated sutures better than uncoated sutures

In this study the capacity of different strains of *S. epidermidis* to adhere to either uncoated or plasma coated sutures was examined. Adhesion of *S. epidermidis* strains 19, 9 and O-47 to sutures was significantly increased in the presence of a human plasma coating (figure 7-7).

Adhesion of S. *epidermidis* to sutures also differed according to the material the suture was constructed from with significantly higher binding to vicryl and silk than to other sutures.

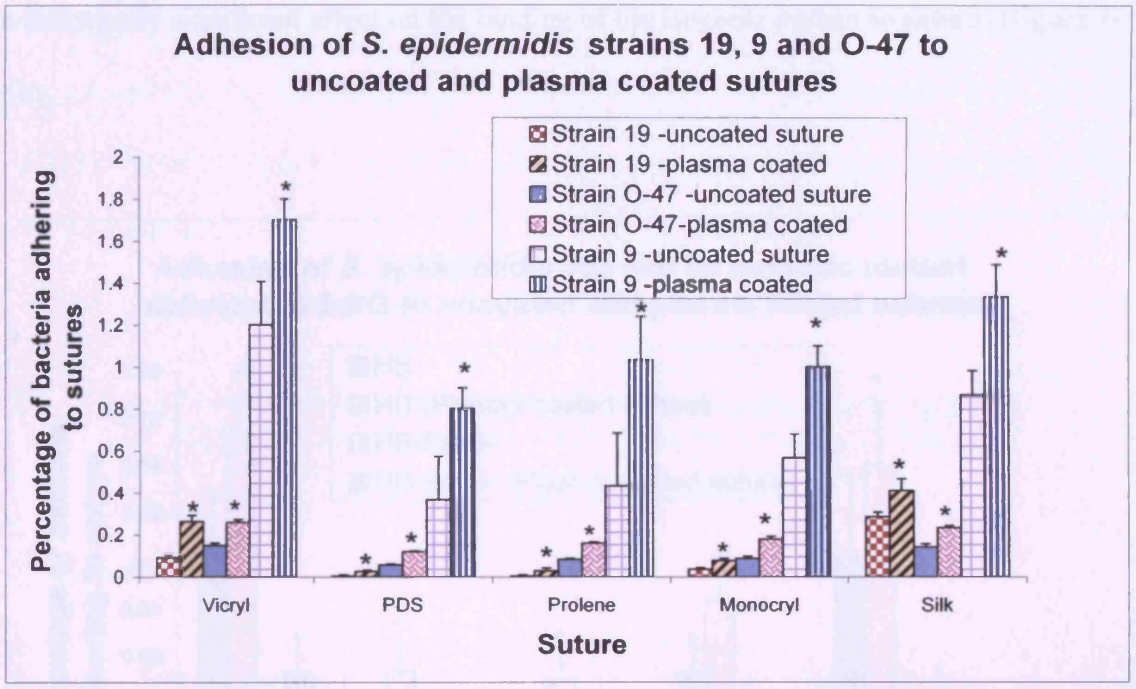

Figure 7-7: Adhesion of *S. epidermidis* strains 19, 9 and O-47 to uncoated or plasma coated sutures. The graph shows the capacity of *S. epidermidis* strains 19, 9 and O-47 to adhere to either uncoated sutures or sutures coated with human plasma. Data are the means and standard deviations of three replicate experiments. * P value < 0.05.

7.4.8 Adhesion of *S. epidermidis* strain HB and its isogenic mutant deficient in SdrG to uncoated or plasma coated sutures

It has been reported that the SdrG protein of *S. epidermidis* may contribute to the initiation of foreign-body infection by allowing bacteria to adhere to biomaterial surfaces that have become coated with host proteins after implantation (Hartford et al., 2001). To

examine the role of *S. epidermidis* SdrG in the adhesion of this bacterium to sutures, *S. epidermidis* strain HB and its isogenic mutant disrupted in SdrG were investigated for their capacity to adhere to uncoated or plasma coated sutures. The isogenic mutant had a reduced capacity to bind to sutures compared to the wild type strain (figure 7-8). Coating sutures with human plasma increased the binding of the wild type strain but did not have a statistically significant effect on the binding of the isogenic mutant to sutures (figure 7-8).

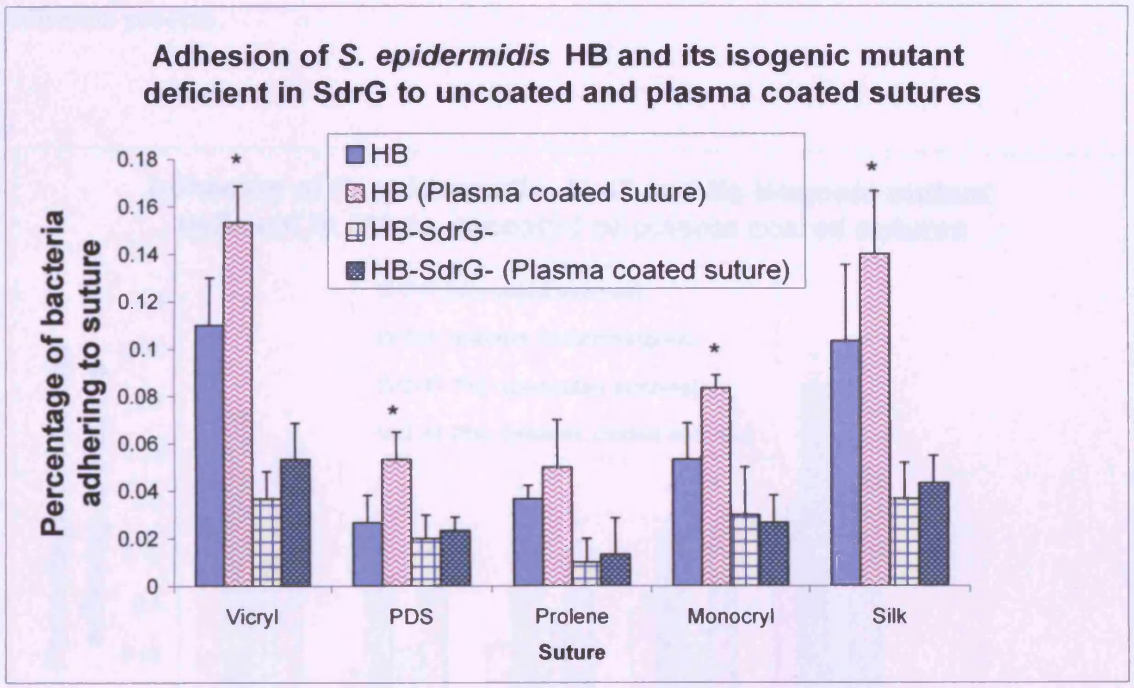

Figure 7-8: Adhesion of *S. epidermidis* strain HB and it isogenic mutant deficient in SdrG to either uncoated or plasma coated sutures. The experiment is a representative of three experiments performed on different occasions. Data are the means and standard deviations of three replicate experiments. * P value < 0.05.

7.4.9 Adhesion of *S. epidermidis* strain O-47 and its isogenic mutant deficient in PIA to uncoated or plasma coated sutures

To examine the role of coating suture with human plasma in the adhesion of *S. epidermidis* strain O-47 and its isogenic mutant deficient in PIA to suture materials, adhesion of these strains was performed using overnight plasma coated sutures. There was a significant increase in the adhesion of *S. epidermidis* strain O-47 and its isogenic mutant deficient in PIA production to all examined plasma coated sutures compared to uncoated sutures (figure 7-9). These findings suggest a role of plasma proteins in the adhesion process.

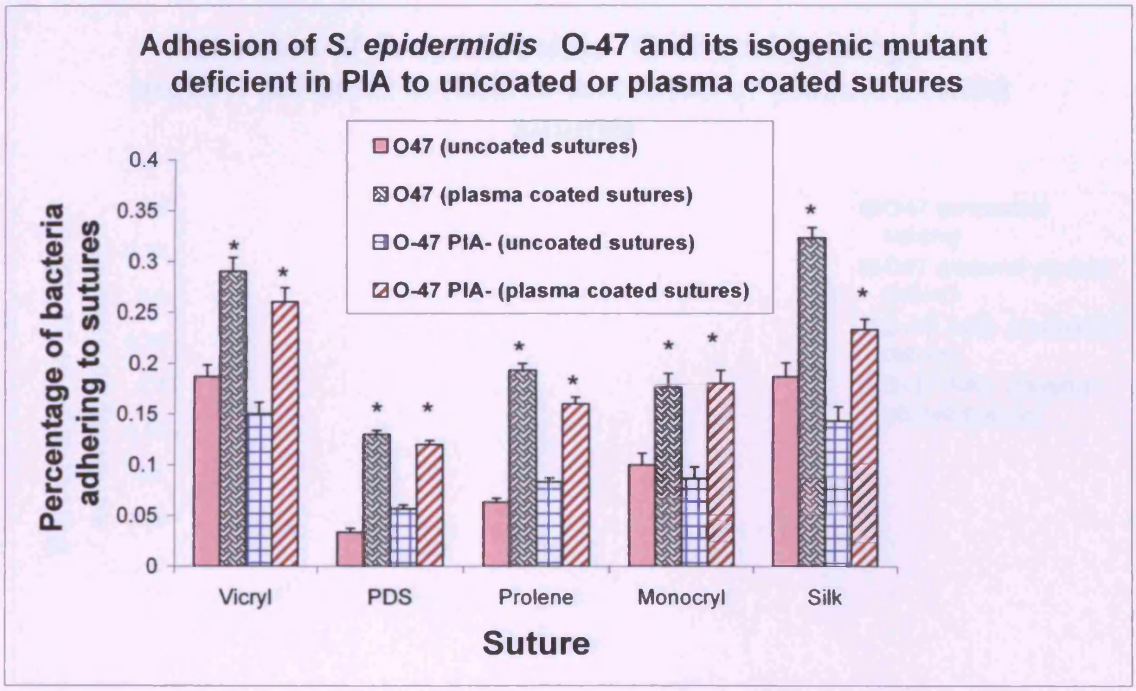

Figure 7-9: Adhesion of *S. epidermidis* strain O-47 and its isogenic mutant deficient in PIA to uncoated or plasma coated sutures. Data are the means and standard deviations of three replicate experiments. The experiment is a representative of three experiments performed on different occasions. * P value < 0.05.

7.4.10 Adhesion of *S. epidermidis* strain O-47 and its isogenic mutant deficient in AtlE to uncoated or plasma coated sutures

To examine the role of *S. epidermidis* AtlE in the attachment of this bacterium to proteins coated sutures, adhesion of *S. epidermidis* strain O-47 and its isogenic mutant deficient in AtlE to overnight plasma coated sutures was investigated. Coating sutures with human plasma did not change the capacity of the isogenic mutant of O-47 deficient in AtlE to adhere to any of the sutures (figure 7-10). These findings suggest that the AtlE of *S. epidermidis* has a role in the indirect attachment of this bacterium to sutures.

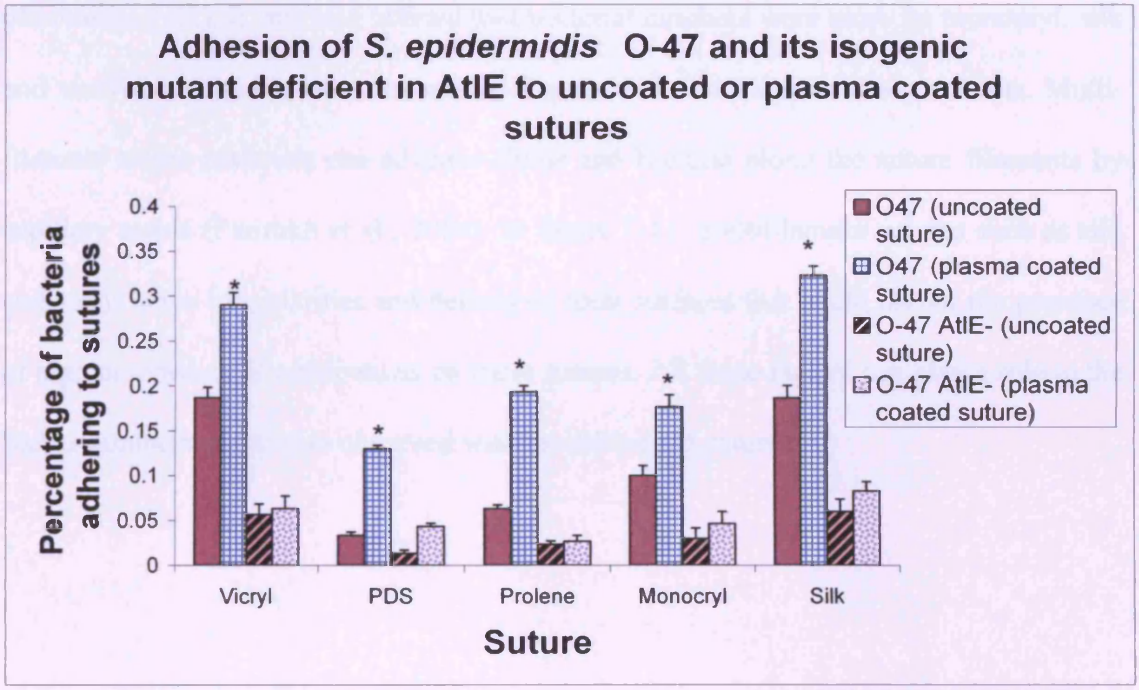

Figure 7-10: Adhesion of *S. epidermidis* strain O-47 and its isogenic mutant deficient in AtlE to uncoated or plasma coated sutures. Data are the means and standard deviations of three replicate experiments. The experiment is a representative of three experiments performed on different occasions. * P value < 0.05.

7.5 Surface scanning electron microscopy of *S. epidermidis* adhered to different sutures

Scanning electron microscopy was used to visualise the adherence of *S. epidermidis* to different surgical sutures (figure 7-11). Examples of the attached *S. epidermidis* to different sutures are indicated by black arrows. The number of suture-bound bacteria on sutures was lower than the number of bacteria obtained by the adhesion assays. This difference could be related to the preparation and manipulation of sutures during the SEM protocol. SEM observations showed two different patterns of bacterial adhesion. Bacteria were attached either as single colonies (monocryl) or group of colonies (silk). It was also noticed that bacterial numbers were more on monocryl, silk and vicryl compared to other sutures and agree with the adhesion assays results. Multi-filament suture materials can advance fluids and bacteria along the suture filaments by capillary action (Parirokh et al., 2004). In figure 7-11, multifilament sutures such as silk and vicryl show irregularities and defects on their surfaces that could favour the presence of high numbers of *S. epidermidis* on these sutures. All these factors can play a role in the higher numbers of bacteria observed with multifilament sutures.

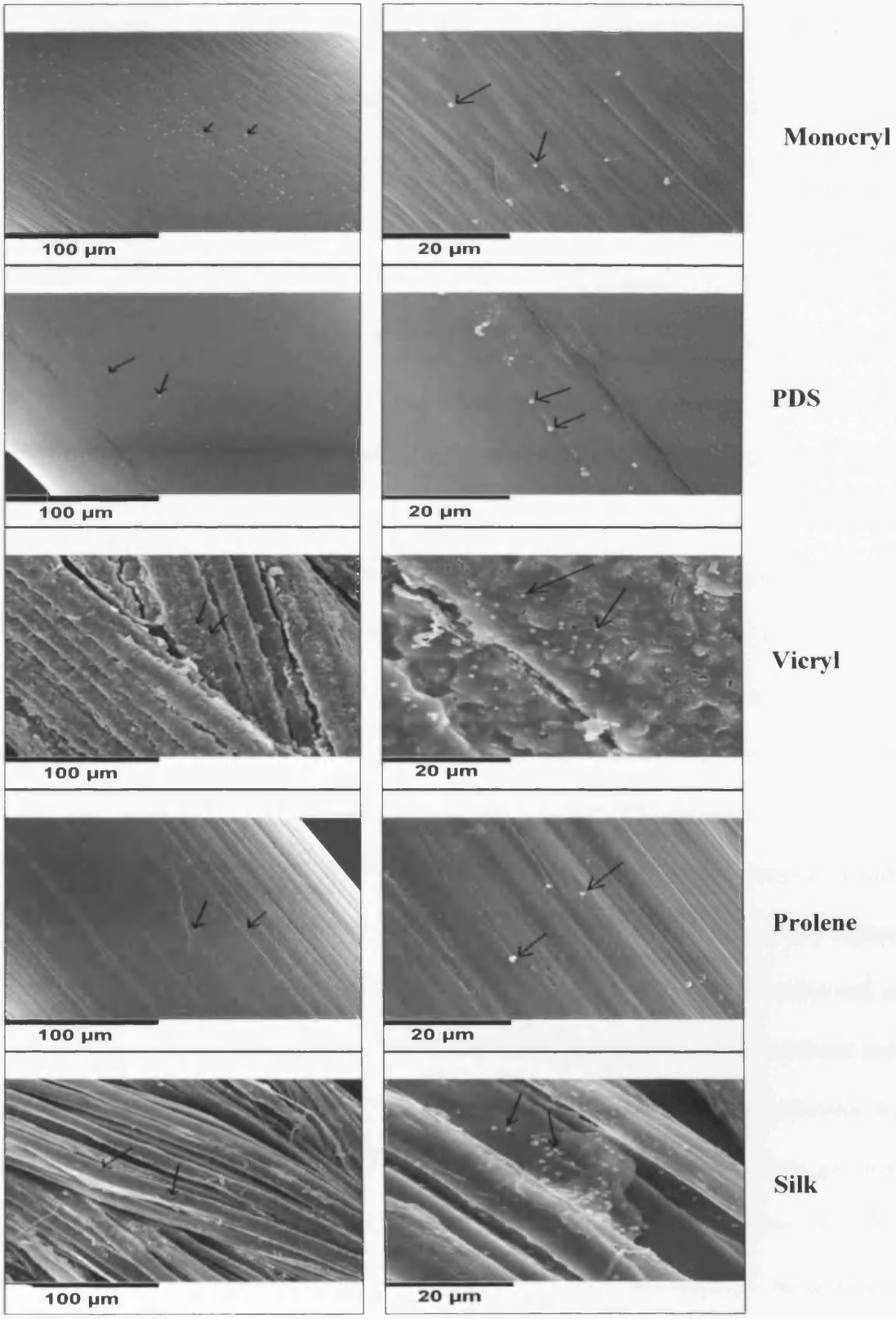

Figure 7-11: Scanning electron microscope image of *S. epidermidis* (arrowed) adhered to monocryl, PDS, vicryl, prolene and silk sutures, magnification 400x and 2000x.

7.6 Discussion

The results from these studies show that *S. epidermidis* strains have different capacities to adhere to surgical suture materials (figure 7-1). There was strain-to-strain variation in bacterial adhesion to sutures. *S. epidermidis* strain NCTC11047 had a better capacity to bind to sutures than strains 19 and RP62A (figure 7-1). Furthermore the material from which the suture was constructed had a significant impact on the adhesion of *S. epidermidis*. Bacterial adhesion is often found to differ between materials with different chemical compositions and/or hydrophobicities (An and Friedman, 1998). Although chemical composition is undoubtedly important, surface texture and roughness are also factors. Scher et al, have questioned the role of the monofilament structure of some sutures to their infection resistance when compared to multifilament sutures (Scher et al., 1985). We have found that fewer bacteria attach to monofilament sutures compared to multifilament sutures (figure 7-1). One possible explanation for this is that multifilament sutures provide a larger surface area to which bacteria can bind. Although some multifilament sutures are easier to handle and provide secure surgical knots, bacterial adhesion to such sutures should be considered in suture selection. Many reports have described the use of sutures coated with antibacterial agents to minimize the risk of infection (Rothenburger et al., 2002). Although this approach is useful, it does have two problems, antibiotic toxicity and the development of resistance to such antibiotics by bacteria. An alternative approach is the development of materials that reduce or even prevent bacterial adherence to sutures. For example as described in a recent report on surfactant polymers designed to suppress *S. epidermidis* adhesion to biomaterials (Vacheethasanee and Marchant, 2000).

Very few *S. epidermidis* virulence factors have been identified and reported in the literature. However a number of adhesins which are believed to be important to the virulence of this organism have been described including the fibrinogen binding protein SdrG (Hartford et al., 2001), polysaccharide intercellular adhesin (Rupp et al., 2001), lipases (Longshaw et al., 2000) and the autolysin AtlE (Rupp et al., 2001). The involvement of these factors in adhesion of this organism to sutures has not been investigated. By using *S. epidermidis* strains with deletions or disruptions in the genes for these virulence determinants, we have examined their role in the adhesion of this bacterium to sutures. The tendency of bacteria to colonise biomaterial surfaces and the ability of some bacteria to produce extracellular polysaccharide matrix and form biofilms has been associated with increased virulence and infections related to these materials. It has been shown that *S. epidermidis* can bind to fibronectin and this can be inhibited by heparin (Arciola et al., 2003). The role of fibronectin in adhesion of staphylococci to biomaterials has also been reported (Delmi et al., 1994). A fibronectin binding protein of *S. epidermidis* has been identified recently (Williams et al., 2002). To examine the role of this fibronectin binding protein in the adhesion of *S. epidermidis* to surgical sutures, isogenic mutants with a disruption at the start of the gene for Embp (HB-Embp3349) and a mutant with a disruption in the 3' prime end of the gene coding for Embp (HB-Embp284335) were used. Disruption at the start of the gene coding for Embp decreased the adhesion of *S. epidermidis* to sutures (figure 7-2). This suggests that the *S. epidermidis* fibronectin binding protein may play a role in the adhesion of this bacterium to surgical sutures. Disruption in the 3' prime end of the gene coding for Embp may not have completely disturbed the gene and the functional domain of the Embp may still have been produced. This may explain the difference in the capacity of the two isogenic

mutants to bind to sutures. The SdrG of *S. epidermidis* has been found to promote adhesion of this bacterium to fibrinogen (Hartford et al., 2001). We examined the role of SdrG in adhesion of *S. epidermidis* to sutures. A *S. epidermidis* isogenic mutant deficient in SdrG, showed a lower level of adhesion to suture materials compared to the parental strain (figure 7-3). This suggests the involvement of SdrG in the adhesion of *S. epidermidis* to suture materials. Another virulence determinant of *S. epidermidis* that has previously been described is the enzyme lipase. Two lipases have been identified in *S. epidermidis*, the GehC and GehD lipases. The GehD lipase has been shown to be a collagen binding adhesin of *S. epidermidis* (Bowden et al., 2002). The role of these two *S. epidermidis* lipases in its adhesion to sutures was investigated. The results showed that the GehD but not the GehC lipase was involved in adhesion of *S. epidermidis* to sutures (figure 7-4). We found that the polysaccharide intercellular adhesin (PIA) was not involved in adhesion of *S. epidermidis* to suture materials (figure 7-5). In 1998 Higashi *et al* found that slime (PIA) does not enhance the adhesion of *S. epidermidis* to polyethylene (Higashi et al., 1998). Although we have found that PIA is not involved in attachment to sutures it does not rule out its possible importance in cell to cell adhesion and biofilm formation in suture related infections. The *S. epidermidis* autolysin AtlE seemed to play an important role in the adhesion of this bacterium to the surgical sutures examined in this study (figure 7-6). Rupp et al, have demonstrated the importance of both PIA and AtlE in a model of catheter related infection (Rupp et al., 2001).

Material related infections are initiated by the bacterial colonization of material surfaces. Current understanding of such processes indicates that bacterial colonization of artificial surfaces may occur via two main mechanisms. These include a direct non-specific

interaction of bacteria with material (uncoated with any host factor), driven by physico-chemical forces or the interaction of specific bacterial surface components, such as adhesins, with host components adsorbed on implanted surfaces (indirect interaction). Sutures were coated with human plasma to examine which of these mechanisms was the most effective in the adhesion of *S. epidermidis* to sutures. Coating sutures with human plasma increased the adhesion of the different *S. epidermidis* strains to suture materials (figure 7-7). This suggests that some factors in the plasma enhanced the adhesion of this organism to sutures and highlights that the indirect adhesion mechanism is more effective than the direct attachment of this bacterium to sutures. Human plasma is composed of a variety of proteins including albumin and fibrinogen. The fibrinogen binding protein, SdrG, of *S. epidermidis* was found to be important in direct attachment of this organism to sutures (figure 7-3). The adhesion of the isogenic mutant of *S. epidermidis* strain HB deficient in SdrG to sutures was not increased when sutures were coated with human plasma (figure 7-8). The SdrG mutant maintained its lower binding capacity to sutures in both attachment mechanisms examined (direct and indirect). These findings suggest that SdrG may be important in the mechanism of attachment of *S. epidermidis* to sutures. The autolysin AtlE was also important in both mechanisms of attachment of *S. epidermidis* to surgical sutures (figures 7-6 and 7-10). Adsorption of plasma proteins on the surface have been reported to affect the adhesion of *S. epidermidis* to different materials. A marked decrease in adhesion on silicone and polyurethane catheters and a marked increase in adhesion on hydrophilic surfaces in the presence of plasma have been reported (Katsikogianni and Missirlis, 2004). In this study, PIA was not found to be important in the direct attachment of *S. epidermidis* to sutures since the adhesion of the PIA mutant strain was similar to the parental strain (figure 7-5). Coating sutures with

plasma increased the adhesion of the *S. epidermidis* isogenic mutant deficient in PIA to these sutures (figure 7-9). The similar behaviour in attachment of both the mutant and the parental strain suggests that PIA is not involved in the adhesion of *S. epidermidis* to sutures. It has been shown that PIA plays a critical role in cell to cell adhesion in case of biofilm formation (Heilmann et al., 1996; O'Gara and Humphreys, 2001). Therefore PIA may be involved indirectly in suture related infections caused by *S. epidermidis*. Biofilm formation on different sutures has been described (Otten et al., 2005).

Higher numbers of bacteria can be seen attached to vicryl and silk sutures when compared to other sutures. Adhesion was either as single cells (prolene, monocryl and vicryl) or clumps of cells (PDS and silk) (figures 7-11). It has been reported that physical characteristics of suture material can affect adhesion of bacteria to sutures (Gabrielli et al., 2001). We found more bacteria attached to multifilament sutures such as vicryl and silk. It has been postulated in a previous report that bacteria can be enclosed in the interstices of the multifilament sutures where they can be protected from the immune system and cause prolong infection (Osterberg, 1983).

These data show that *S. epidermidis* strains have different capacities to adhere to different sutures and some of the virulence determinants of this bacterium were involved in the process of adhesion. Adhesion of bacteria should be taken in consideration in the selection of suture material to decrease the risk of suture-related infection.

7.7 Conclusions

This study demonstrates the ability of *S. epidermidis* to adhere to different surgical suture materials. *S. epidermidis* virulence determinants such as the GehD lipase, SdrG, the fibronectin binding protein Embp and the autolysin AtlE are involved in the adhesion of this bacterium to these sutures. Coating sutures with human plasma increased the adhesion of *S. epidermidis* to surgical sutures. Infection is one of the most serious complications associated with foreign devices and bacterial adhesion to sutures should be considered when selecting suture materials to reduce the risk of wound infection and subsequent complications. The autolysin AtlE plays a critical role in the adhesion of *S. epidermidis* biomaterials and in biofilm formation. Inhibitors of the *S. epidermidis* autolysin AtlE may be useful coating agents to prevent bacterial adhesion to sutures. The use of surgical sutures which resist bacterial adhesion could also help in reducing the risk of suture-related infections. As suture material properties have the potential to influence wound healing negatively and may increase the risk of infection a thorough understanding of suture-bacterial interaction appears essential to optimize surgical management and reduce the risk of infection.

Chapter 8

Chapter 8

Discussion

The work presented in this thesis explores the interaction of *S. epidermidis* with abiotic surfaces and host cells.

8.1 Introduction

The reader of this project may question why we have studied the interaction of *S. epidermidis* with osteoblasts, epithelial cells and sutures. In order to understand the reason it is useful to consider the clinical situation in which a patient undergoes a surgical procedure involving the implantation of a prosthetic device. In all patients undergoing surgery an intravascular catheter needs to be introduced through the skin as a part of the patient's preparation for surgery. Prosthetic devices can be either embedded completely in the body or have an external part that passes through the host tissue such as some dental and orthopaedic implants. The surgical procedure will also involve the use of surgical sutures which provide one of the main methods of closing surgical wounds. Proper closure and stabilisation of wound margins are critical events that may influence the success of any surgical procedure. However the presence of foreign material in the wound significantly enhances the susceptibility of host tissues to infection (Leknes et al., 2005). In addition, sutures may give rise to inflammatory responses, reduce resistance to infection and ultimately impair wound healing . Physically, sutures may also serve as a pathway for bacterial entry into a surgical wound which may be enhanced by the capillary action of the suture material (Chu and Williams, 1984; Leknes et al., 2005). Other sources of bacterial contamination such as a contaminated implant or endogenous flora have been reported (Haas et al., 2005).

The situation is more complicated in the case of infected wounds where sutures can increase the risk of infection despite thorough irrigation (Mehta et al., 1996). Moreover, only low numbers of bacteria are required to establish an infection in the presence of a biomaterial (Merritt et al., 1999). The development of infections in incisional wounds is one serious complication that may occur. Surgical infections may not only affect wound healing and implanted device failure but also may induce life-threatening clinical situations particularly in immunocompromised patients (Leknes et al., 2005). The presence of a source of bacteria can lead to contamination of the surrounding tissues. Bacteria attached to sutures can reach deeper tissue such as bone either through the surgical wound, a contaminated device or a haematogenous route.

Internalisation of bacteria by host cells is a recently described phenomenon. Once the bacteria reach non-phagocytic host cells there is the possibility that these cells can internalise the microbes. Internalisation of *S. epidermidis* by epithelial cells may have an effect on surgical wound healing. If *S. epidermidis* gains access to deeper tissues such as bone, there is also the possibility that these bacteria could be internalised by bone cells. Internalisation of bacteria by host cells has been suggested to protect the bacteria from host defences and antibiotic therapy and may be a possible explanation of the chronicity and recurrence of some diseases such as osteomyelitis (Haas and McAndrew, 1996; Ciampolini and Harding, 2000). It has been shown that *S. epidermidis* can survive in phagocytic host cells such as macrophages and this will add to the complexity of the disease caused by this bacterium (Boelens et al., 2000).

Figure 8-1 shows some possible interactions of *S. epidermidis* with implanted devices and host cells. Adhesion of *S. epidermidis* to suture materials and the capacity of this bacterium to be internalised by host cells may be possible factors that affect the outcome of surgery. Research on *S. epidermidis* has gained a greater interest during the last decade as this organism has become recognised as one of the most important pathogens in device related infections.

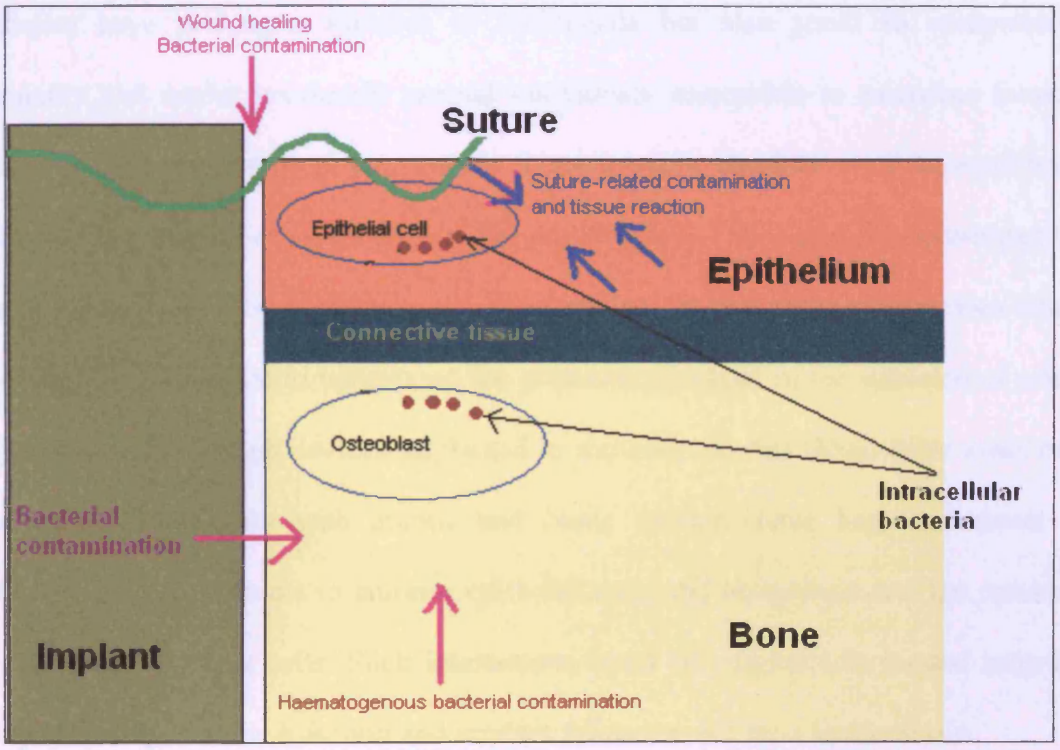

Figure 8-1: Possible interaction of *S. epidermidis* with implanted devices and host cells.

S. epidermidis is a skin commensal and is considered to be the leading cause of nosocomial infections and infections of indwelling medical devices (Vuong and Otto, 2002). All implantable devices which are introduced into the body have the risk of

becoming contaminated by *S. epidermidis*. Although there have been advances in aseptic techniques infections do still occur. In the case of hip arthroplasty surgical site infections are estimated to be around 2% (Kaltsas, 2004). The majority of biomaterial-associated infections, 40% to 75%, are caused by the relatively nonpathogenic coagulase-negative staphylococci, especially *S. epidermidis*. Some of these infections are difficult to treat and require prolonged hospital stays and sometimes the removal of the implanted device. Medical advances have also given rise to new infection problems: organ transplantation, invasive surgery, implantation of prosthetic devices, and the use of immunosuppressive therapies have prolonged survival of individuals but also result in compromised immunity and render previously normal individuals susceptible to microbes formerly considered to be pure saprophytes such as *S. epidermidis*. Breakdown of the equilibrium between the host and bacterium leads to the proliferation of endogenous micro-organisms and/or contamination by exogenous micro-organisms. To prevent or treat device-related infections, it is essential to understand the processes involved in the adhesion of micro-organisms to the foreign devices implanted in the body. In this thesis three areas of *S. epidermidis* interactions with abiotic and biotic surfaces have been examined; the adhesion of *S. epidermidis* to sutures, epithelial cells and osteoblasts and the uptake of this bacterium by host cells. Such interactions could be important to wound infection, delayed healing, chronic infection and implant failure caused by *S. epidermidis*.
Aspects of the thesis and its implications will be discussed in the following sections.

8.2 Internalisation or invasion

Although the words internalisation and invasion are used interchangeably in the literature in regard to bacterial host cell interaction, they do actually have different

meanings. The word invasion means a hostile entrance into a domain of another, so bacterial invasion means that the bacteria are responsible for the event without necessarily requiring the help of the host cell, which may even offer resistance to this entry i.e. it is a forced entry into the host cell. On the other hand the word internalisation means to take in or incorporate and here the host cells are responsible for the uptake event and allow the bacteria to enter into the cytoplasm by utilising different mechanisms such as rearrangement of microfilaments which are part of the cellular cytoskeleton. In order to apply the correct word to the uptake of bacteria by host cells a detailed understanding of the mechanisms involved in the process are required. The entry of dead bacteria into bone cells which was described by Hudson *et al.* in 1995 is an example of internalisation. In some cases a combination of invasion and internalisation occurs in the uptake process in which the bacteria and the host cells both take part.

8.2.1 Internalisation of *S. epidermidis* by host cells

In chapters 3 and 6, the ability of *S. epidermidis* to be internalised by osteoblasts and epithelial cells was examined. *S. epidermidis* has the capacity to be internalised by osteoblasts and epithelial cells. It has been suggested that bacteria can evade humoral immunity by hiding in the cytoplasm of host cells. The intercellular persistence of bacteria may be one of the explanations for why some bacterial infections are chronic and difficult to eradicate. The intracellular persistence of *S. epidermidis* in macrophages has been suggested to be one of the mechanisms responsible for inflammatory reactions in the pericatheter area (Boelens et al., 2000). Another study has shown that *S. epidermidis* can be taken up by bovine mammary epithelial cells (Almeida and Oliver, 2001). In this thesis it was considered important to examine the interaction of *S. epidermidis* with

epithelial cells, where wound healing occurs and with osteoblasts where biomaterial-bone integration is considered a critical stage in the osseointegration of implants. It was shown that different strains of *S. epidermidis* had different capacities to be internalised by host cells with some strains having no capacity to be internalised by host cells.

Microbes can employ a variety of mechanisms to enter eukaryotic cells, but most modes of entry require some rearrangement of the host cytoskeleton. Many bacterial pathogens, such as *Salmonella* and *Yersinia* species, require an intact actin network for the initial entry process (Dramsi and Cossart, 1998). This entry event is inhibited by the microfilament disrupter cytochalasin D. In the case of *S. epidermidis* when internalisation by osteoblasts and epithelial cells was examined, cytochalasin D had different effects. The capacity of some *S. epidermidis* strains such as strain 19 and NCTC11047 to be internalised by host cells was increased in the presence of cytochalasin D (chapter 3). The uptake of other strains of *S. epidermidis* such as strain HB was inhibited by cytochalasin D but not to the extent that has been reported for the internalisation of *S. aureus* by host cells (Jevon et al., 1999). The use of a panel of inhibitors in chapters 3 and 6 showed that *S. epidermidis* is internalised by osteoblasts and epithelial cells via a receptor mediated pathway. The findings also suggested that *S. epidermidis* utilises different pathways in gaining access to host cells some of which may not depend on actin filament polymerisation. Similar findings on the role of host cell actin in the uptake process have been obtained with other bacteria such as *A. actinomycetemcomitans* (Brissette and Fives-Taylor, 1999). The exact mechanism behind the increase in the number of intracellular bacteria in the presence of cytochalasin D is not clear but could be due to the change in

cell morphology which was noticed during experimentation (figure 3-13) or exposure of the basolateral cell surface as suggested by Wells et al (Wells et al., 1998).

Microtubules played some role in the internalisation of *S. epidermidis* by epithelial cells but were not involved in the internalisation of this bacterium by osteoblasts. These findings suggest that epithelial cells and osteoblasts utilise different pathways to internalise *S. epidermidis*. Certain strains of *Campylobacter jejuni* also exhibit actin independent entry into mammalian cells and their entry is instead mediated by host microtubules (Brissette and Fives-Taylor, 1999).

S. epidermidis does not produce many toxins and tissue damaging enzymes in contrast to *S. aureus*. In fact few *S. epidermidis* virulence factors have been identified and their specific role in the virulence of the organism needs to be investigated further (Vuong and Otto, 2002).

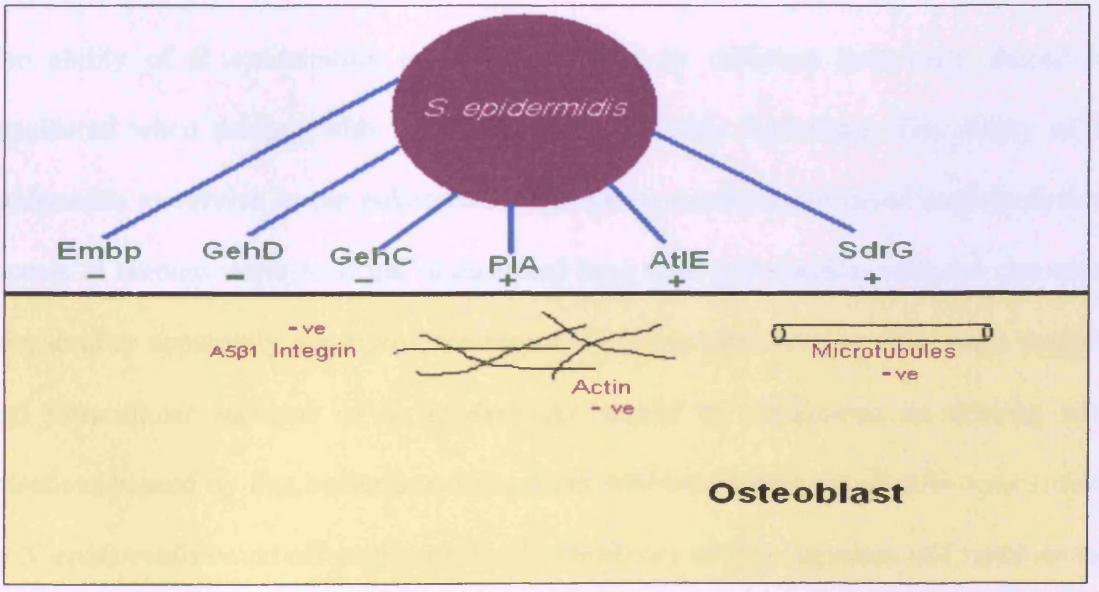

Figure 8-2: *S. epidermidis* and host factors involved in internalisation of *S. epidermidis* by osteoblasts. (+ indicates possible role, - indicates no role).

In chapters 4 and 6 the role of some *S. epidermidis* virulence determinants in the internalisation of this bacterium by osteoblasts and epithelial cells was examined. Figure 8-2 shows some of the virulence factors that were examined in this thesis. Factors such as SdrG and AtlE were important in the internalisation of some strains of *S. epidermidis* by osteoblasts.

Internalisation of *S. aureus* by epithelial cells has been shown to induce expression of the inflammatory cytokines such as tumor necrosis factor alpha and interleukin-1β by host cells and also to induce host cell apoptosis (Wesson et al., 2000). Cytokine production has been shown to activate the immune system and cause local tissue damage. However as demonstrated in chapter 5, *S. epidermidis* did not induce the production of a number of cytokines tested or induce apoptosis of osteoblasts. This data may explain why infections due to *S. epidermidis* are in most cases sub-acute.

The ability of *S. epidermidis* to be internalised by different host cells should be considered when dealing with infections caused by this bacterium. The ability of *S. epidermidis* to survive in the eukaryotic intracellular environment could explain several aspects of chronic staphylococcal disease and long term colonisation with the chance of relapse after apparently successful treatment. The data presented in this thesis suggest that intracellular survival of *S. epidermidis* should be considered in dealing with infections caused by this bacterium. Long term antibiotic treatment of infections caused by *S. epidermidis* could affect the antibiotic sensitivity of this organism and result in the emergence of antibiotic resistance strains which increase the difficulty of dealing with such infections.

8.3 Adhesion to surgical sutures

Sutures are considered the most common foreign devices introduced into the body. However, little research has been conducted to examine the role of bacterial adhesion to these devices and the risk of infection. Even though there have been advances in aseptic techniques, biomaterial-related infections still occur. Bacterial infections are a serious complication following orthopaedic implant surgery that can usually only be cured by removing the implant (Hendriks et al., 2004). Many strategies have been developed to reduce the risk of device related infections. Some research has focused on coating sutures with antimicrobials or changing the surface properties of the material itself. However, incorporating antimicrobials into implant material has also proven largely unsatisfactory in clinical practice, despite encouraging findings *in vitro* (Domenico et al., 2004). New approaches are needed to prevent colonisation of prosthetic surfaces. One promising advance is the use of agents that interfere with quorum sensing, or cell-to-cell communication in bacteria which is part of the multicellular strategy that promotes biofilm formation. In chapter 7 the ability of *S. epidermidis* to adhere to surgical sutures as well as the role of some of the known *S. epidermidis* virulence determinants in the process of adhesion was examined. *S. epidermidis* had the capacity to adhere to different sutures. Figure 8-3 shows the virulence factors that were examined for their role in the adhesion process. Virulence factors such as SdrG, AtlE, Embp and the GehD lipase were found to play a role in the adhesion of *S. epidermidis* to surgical sutures (chapter 7). These factors could be targets for possible prevention options such as coating sutures with agents that inhibit their adhesive capacity and thus reduce the risk of suture-related infection.

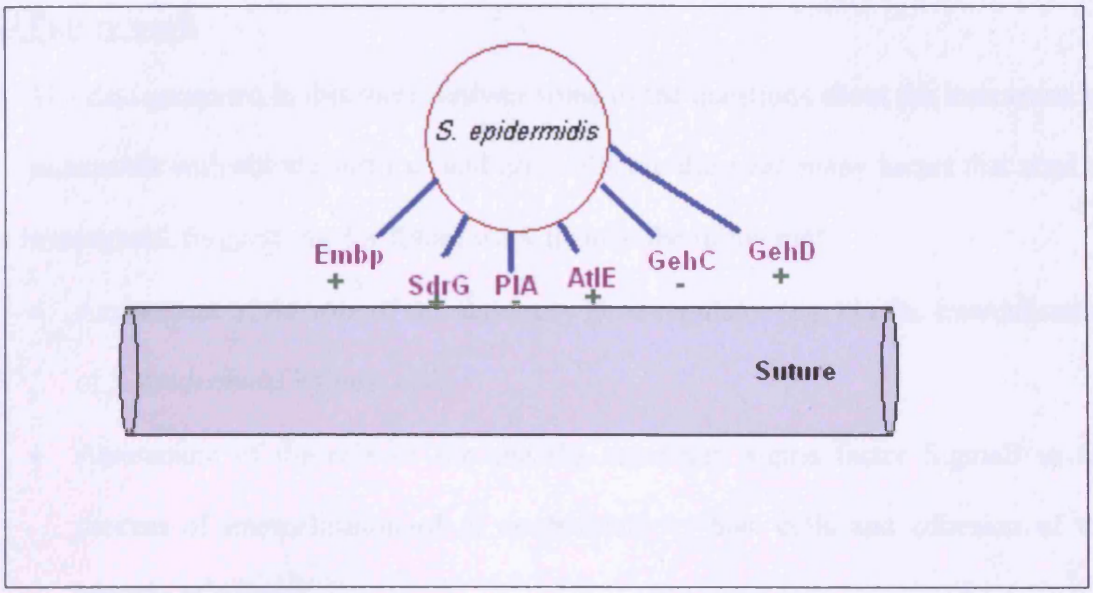

Figure 8-3: *S. epidermidis* virulence factors involved in adhesion to surgical sutures. (+ indicates possible role, - indicates no role).

In conclusion, the data presented in this thesis on the capacity of *S. epidermidis* to interact with abiotic surfaces and host cells should be considered when dealing with infections caused by this organism and their prevention and treatment. Targeting specific staphylococcal virulence factors may be a novel approach for the treatment of staphylococcal infections.

8.4 Future work

The data presented in this thesis answer some of the questions about the interaction of *S. epidermidis* with abiotic surfaces and host cells but there are many issues that need to be investigated. Suggestions for future work include the following:

- Assessment of the role of the accessory gene regulator (*agr*) in the internalisation of *S. epidermidis* by host cells
- Assessment of the role of *sar* and the secondary sigma factor SigmaB in the process of internalisation of *S. epidermidis* by host cells and adhesion of the bacteria to sutures.
- Investigation of the reasons for the increased numbers of intracellular *S. epidermidis* in the presence of cytochalasin D.
- *In vivo* assessment of the internalisation of *S. epidermidis* by host cells.
- Construction of a *S. epidermidis* gene library or transposon mutant libraries to identify the bacterial gene(s) involved in internalisation of this bacterium by host cells
- Investigation of the adhesion of *S. epidermidis* to other types of sutures.
- Randomised controlled trials to assess of the risk of infection from bacteria adhered to surgical sutures

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